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Ciências

Proteomic study of the therapeutic effect of a DNA vector on cervical cancer cells

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Resumo Alargado

O cancro é um dos principais problemas de saúde, sendo responsável por mais de 8 milhões de mortes todos os anos, a nível mundial. O cancro do colo do útero é a 3ª malignidade mais frequente, sendo a 4ª causa de morte nas mulheres a nível mundial. A infeção persistente por HPV de alto risco é o fator principal para o desenvolvimento de cancro do colo do útero, dado que as oncoproteínas E6 e E7 são capazes de promover o desenvolvimento cancerígeno através da degradação e inibição das proteínas supressoras de tumor, p53 e pRb, respetivamente. A terapia génica é uma estratégia promissora no tratamento de doenças adquiridas e/ou desordens genéticas, tendo como objetivo a entrega de material genético em células ou tecidos alvo, de forma a induzir um efeito terapêutico. O DNA minicircular (mcDNA) é um novo produto biofarmacêutico, que se caracteriza por ser apenas constituído pela unidade de transcrição eucariota, aumentando a sua segurança e efeito terapêutico, sendo obtido através da recombinação intramolecular do plasmídeo parental. Além disso, tem-se atribuído aos microRNAs (miRs) um papel regulador da carcinogénese, por exemplo, está descrito que o miR-375 possui a capacidade de silenciar as oncoproteínas E6 e E7.

Desta forma, este trabalho tem como objetivo a produção e purificação de vetores de mcDNA que codifiquem para os genes primiR-375 e p53, de forma a silenciar as oncoproteínas E6 e E7 e reestabelecer os níveis das proteínas supressoras de tumor p53 e pRb nas células do cancro do colo do útero.

A purificação dos vetores de mcDNA foi efetuada utilizando colunas de sefarose (Sephacryl S-1000 SF) de cromatografia de exclusão molecular, demonstrando que para o vetor de tamanho mais pequeno, mcDNA-primiR-375, uma coluna de 106 mL permitiu uma recuperação eficiente de frações cromatográficas compostas apenas por mcDNA. No entanto, para os vetores de maior tamanho, mcDNA-p53 e mcDNA-primiR-375+p53, foi necessário explorar os parâmetros que afetam a separação das moléculas das amostras a purificar, optando-se por utilizar uma coluna com o volume de 180 mL, permitindo assim, à semelhança do mcDNA-primiR-375, recuperar frações compostas apenas por mcDNA. Paralelamente foram realizados ensaios de citotoxicidade em fibroblastos humanos e células CaSki, revelando que nenhum dos vetores é tóxico nos fibroblastos humanos e que o mcDNA-primiR-375+p53 apresentou a menor viabilidade celular em células CaSki. Para além disso, os resultados do ensaio de proliferação realizado em células CaSki demonstraram que o número de células viáveis diminui nas células transfectadas com os vetores de DNA, corroborando os ensaios de citotoxicidade realizados nesta mesma linha celular cancerígena. A realização do ensaio de Western-Blot confirmou o restabelecimento dos níveis da proteína supressora de tumor p53 após 48 horas de transfecção com mcDNA-p53 e mcDNA-primiR-375+p53 em células CaSki.

Os resultados obtidos nos em ensaios in vitro desenvolvidos neste trabalho, sugerem que o uso de vetores de DNA que codifiquem para mais do que um gene com finalidade terapêutica, como é exemplo o mcDNA-primiR-375+p53, permitiram observar efeitos mais significativos, sugerindo assim que este tipo de vetores podem ter uma ação terapêutica mais eficiente.

Keywords

Cervical cancer; Gene therapy; minicircle DNA; miR-375; p53; pRb

Abstract

Cancer is a major global health problem, accounting for more than 8 million deaths/year worldwide. In particular, cervical cancer is the 3rd most common malignancy and 4th cause of death among women globally, being a persistent High Risk-HPV infection the main factor for cervical cancer development. E6 and E7 HPV oncoproteins are responsible for cancer development by degrading and inhibiting p53 and pRb tumour suppressor proteins, respectively. Moreover, microRNAs (miRs) have been found to regulate tumorigenesis. In fact, miR-375 has the ability to silence HPV E6 and E7 oncoproteins. Gene therapy is a promising strategy to treat acquired diseases and/or genetic disorders, aiming to deliver genetic material into target cells or tissue, expressing it to induce a therapeutic effect. Minicircle DNA (mcDNA) is a new biopharmaceutical product only composed by the eukaryotic transcription unit, improving its safety and therapeutic effect, obtained through intramolecular recombination of the parental plasmid.

Thus, this work aims to produce and purify a mcDNA vector encoding primiR-375 and p53 genes to silence E6 and E7 oncoproteins and re-establish p53 and pRb tumour suppressor levels on cervical cancer cells.

The purification of mcDNA vectors was performed using size exclusion chromatography sepharose columns (Sephacryl S-1000 SF), showing that for the smallest vector, mcDNA-primiR-375, a 106 mL column allowed the efficient recovery of chromatographic fractions composed only with mcDNA. On the other hand, for the biggest mcDNA vectors, it was necessary to exploit the parameters affecting the sample molecules separation, choosing to use a column with 180 mL of volume, allowing, similarly to the mcDNA-primiR-375 purification, recover fraction only composed with mcDNA. Simultaneously, cytotoxicity assays on human fibroblasts and CaSki cells were performed, confirming that none of the vectors were toxic to the fibroblasts and the mcDNA primiR-375+p53 presented the lower cell viability for CaSki cells. In addition, the proliferation assay results performed on CaSki cells show that number of viable cells decreases for the mcDNA vectors transfected cells, corroborating the cytotoxicity assays results for this cell line. Western-Blot confirmed the re-establishment of tumour suppressor proteins levels after 48h of transfection using mcDNA-p53 and mcDNA-primiR-375+p53.

Overall, these results suggest that the use of DNA vectors encoding for more than one therapeutic gene, for example, the mcDNA-primiR-375+p53, allowed to achieve, on in vitro assays, results suggesting a possible faster therapeutic action, thus demonstrating that they may revolutionize their application in targeted therapies.

Keywords

Cervical cancer; Gene therapy; minicircle DNA; miR-375; p53; pRb

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List of Abbreviations

2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis

AC: Affinity chromatography

AEC: Anion exchange chromatography

AGO: Argonaute

AIF: Apoptosis inducing factor

bac-ORI: Bacteria replication origin

Bax: Bcl-2-associated X protein

Bcl: B-cell lymphoma

bHLH: Basic helix-loop-helix

bp: Base pair

BSA: Bovine serum albumin

CaSki: Cervical cancer cell line

CDK: Cyclin-dependent kinase

CEC: Cation-exchange chromatography

Da: Dalton

DD: Death domain

DISC: Death-inducing signalling complex

DMEM-F12: Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture

DNA: Deoxyribonucleic acid

E. coli: Escherichia coli

ECL: Enhanced Chemiluminescence

EDTA: Ethylenediamine Tetraacetic Acid

EGFR: Epidermal Growth Factor Receptor

EGTA: Ethylene Glycol Tetraacetic Acid

EMA: European Medicines Agency

FADD: Fas-Associated Death Domain

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

g: G force

gDNA: Genomic DNA

HDL: High-Density Lipoprotein

hFIB: Human Fibroblasts cell line

HIC: Hydrophobic Interaction Chromatography

HPV: Human Papillomavirus

HR-HPV: High-Risk Human Papillomavirus

HSPGs: Heparin Sulphate Proteoglycan

IEX: Ion Exchange Chromatography

IRF-3: Interferon Regulatory Factor 3

Kb: Kilobase

Kbp: Kilo basepair

kDa: Kilo Dalton

L1: Major capsid protein

L2: Minor capsid protein

LB: Luria-Bertani

LCR: Long Control Region

LDL: Low-Density Lipoprotein

LR-HPV: Low-risk Human Papillomavirus

m/z: Mass-to-charge ratio

MALDI: Matrix Assisted Laser Desorption Ionization Time of Flight

MAML1: Mastermind-like protein 1

mcDNA: Minicircular DNA

miRNA: Micro RNA

MOMP: Mitochondrion Outer Membrane Permeabilization

MOPS: 3-(N-morpholino)propanesulfonic acid

mP: Mini plasmid

mRNA: Messenger RNA

MW: Molecular Weight

NCBI: National Center for Biotechnology Information

NCR: Non-coding region

OD: Optical Density

ORF: Open Reading Frames

Ori: Origin of replication

PBS: Phosphate buffer solution

pDNA: Plasmid DNA

pfu: Plaque-forming unit

pI: Isoelectric point

PMSF: Phenylmethylsulfonyl fluoride

PP: Parental Plasmid

pRb: Retinoblastoma protein

pre-miRNA: Precursor miRNA

pri-miRNA: Long primary transcripts of miRNA

PTM: Post-translational modifications

PVDF: Polyvinylidene difluoride

RBC: Carboxyl-terminal domain

RF: Radiofrequency

RNA: Ribonucleic acid

rpm: Revolutions per minute

RPMI: Roswell Park Memorial Institute

sc: Supercoiled

SDS: Sodium Dodecil Sulphate

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SEC: Size Exclusion Chromatography

SiHa: Cervical cancer cell line

Smac: Second mitochondria-derived activator of caspases

SNS: Portuguese National Health System

TAE: Tris-acetate-EDTA

TB: Terrific Broth

TBS: Tris-Buffered Saline

TBS-T: Tris-Buffered Saline 0.1% Tween 20

TGF-β: Transforming Growth Factor Beta

TNF: Tumour Necrosis Factor

TOF: Time of Flight analyzer

TRADD: TNRF1-Associated Death Domain

TRAIL: TNF-Related Apoptosis Inducing Ligand

Tris: Tris(hydroxymethyl)aminomethane

Tween 20: Polysorbate 20

UTR: Untranslated Region

VEGF: Vascular Endothelial Growth Factor

List of Scientific Communications

Oral communication

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Chapter I – Introduction

1.1 Cancer

Cancer is a major global health problem accounting, annually, for more than eight million deaths globally, being a complex multifactorial disease that comprises changes in the genome, orchestrated by host and environmental interactions[1]. The hallmarks of cancer are self-sufficiency in growth signals, the ability for tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evasion of apoptosis[1]. The tumour microenvironment, which is composed of various non-malignant cells expressing several regulatory proteins, as well as the extracellular matrix, plays a pivotal role in the initiation and progression of cancers[2].

The process of cancer initiation is irreversible and results from the normal cell exposure to physical, chemical and/or biological agents capable of promoting genetic damages, leading to genomic instability[3], being characterized by the increase in cell plasticity and entropic status, resulting in clonal expansion of mutated cells[4].

The cervical cancer is the third most common malignancy and the fourth cause of death in the world[5]. According to data from the National Health System (SNS) in Portugal, cancer incidence has been growing at a constant rate of 3% per year, mainly associated with the aging of the population. To the date, each year 50000 new cases of cancer are diagnosed, and the mortality is 152.6/100000. Particularly for cervical cancer in Portugal, 1000 new cases per year are diagnosed and, in comparison to other countries in Europe, Portugal has an incidence and mortality rates very favourable. It is important to note that Portugal has a free vaccine against HPV on their national vaccination program since 2008, and the most recent statistical analysis point that 90% of the women population under the age of 27 are vaccinated.

1.1.1 Human papillomavirus (HPV)

Human papillomavirus (HPV) is the etiological agent of common dermatologic and sexually transmitted diseases[6]. A persistent infection with HPV is the main risk factor for the development of cervical cancer[7] and even though being discussed as a sexually transmitted disease[7], evidences suggest that the HPV can be vertically transmitted, as viral DNA sequences were identified in the oral cavity of new-borns, breast milk, amniotic liquid, placenta, umbilical blood and in spermatozoa[8,9].

The infections caused by HPV, according to their oncogenic potential and prevalence in cancer can be classified in low-risk HPVs (LR-HPV) and in high-risk HPVs (HR-HPV)[7]. High-risk HPVs are the causative agents of cervical carcinoma since, it has been demonstrated that E6 and E7 viral oncoproteins are able to promote cancer development by principally targeting and inhibiting p53 and pRb tumour suppressor proteins, respectively[10].

More than two hundred different HPV types have been identified and classified into five genera: α, ß, μ and ν[6]. The HPV belonging to the α genus has proven oncogenic potential, a well-established role in cervical carcinoma[11]. The International Agency for Research on Cancer (IARC) classified 12 different HR-HPV types as carcinogenic to humans: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59[12]. The most common types of HPV are the 16 and 18 most frequently found in cervical cancer, in 60% and 15% of the cases, respectively, both preventable by vaccination [6,7,12]. Moreover, HR-HPV are related to other cancers as vulvar, vaginal, anal, penile and oropharyngeal squamous cell carcinoma[7]. An HPV phylogenetic tree has been designed that groups the different HPV types into genera based on the homologous nucleotide sequence of the major capsid protein, L1[13].

1.1.2 Molecular biology

Human papillomavirus belongs to the *Papilomaviridae* family[9], capable of infecting epithelial cells of the skin, oral and genital mucosa[6,12]. HPVs are small non-enveloped viruses with a double-stranded circular DNA with a length of about 8 Kb[7,9]. The viral particles of HPV (virions) show a conserved icosahedral morphology[6,9], a molecular weight of $5x10^6$ Da[14] and a diameter of 50-55 nm[9].

The viral genome is divided into three regions (Figure 1):

i. early (E) region, that contains E1, E2, E4, E5, E6 and E7 genes, associated with the viral replication [6,7,9];
ii. late (L) region, which encodes the major (L1) and minor (L2) capsid proteins[6,9,15];
iii. non-coding region (NCR), located between the L1 and E6 containing the open reading frames (ORFs)[9].

Human papillomavirus include genes that express non-structural proteins needed for DNA replication, transcription or viral assembly and release[6]. Even tough non-coding, the NCR also known as long control region (LCR), is a fragment of the HPV genome of approximately 900 bases[7], contains most of the regulatory elements involved in viral DNA replication and transcription, including the replication origin (*Ori*)[12].

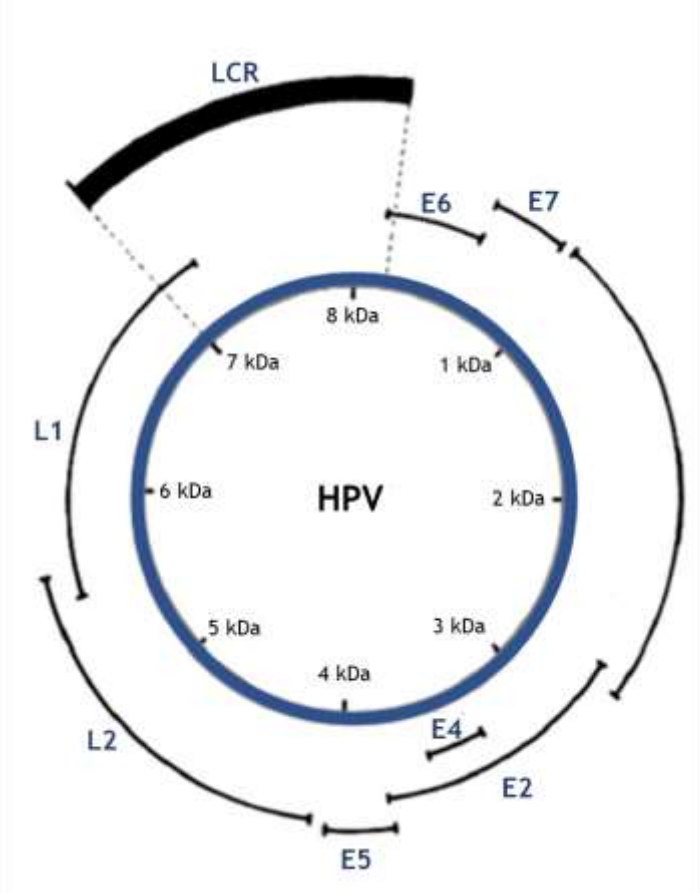

Figure 1. Human Papillomavirus genome representation (adapted from Tomassino, 2014)[12].

The replication of HPV depends upon the host cell differentiation program[7]. E1 and E2 are expressed at the beginning of the infection, bind to the LCR and participate in viral replication and transcription[7]. E4 is expressed mainly during later stages of infection, contributes to the amplification of the viral genome and disrupts the cellular keratin network, facilitating virus release[7]. E5 inhibits maturation of endocytic vesicles from early to late endosomes[7]. E6 and E7 are the main viral oncoproteins, that as multifunctional proteins, regulate the cell cycle through their interaction with a set of cellular proteins, being within the most relevant the tumour suppressor proteins p53 and pRb, respectively[16,17]. E6 and E7 oncoproteins target numerous cellular proteins and thus promote cell cycle progression, apoptosis inhibition, DNA damage and evasion of the host immune response[18].

1.1.3 Infection

As the HPV is not capable of self-replicate (this virus does not express polymerases), during the amplification process, the early proteins interact with the host cell, inducing the S phase entry as obtaining mechanism of DNA polymerases[19,20]. However, this proliferative action can lead to DNA replication stress[9] and, as a consequence, to numerical and structural instability, that can drive to cancer initiation[9].

The life cycle of HPV is intimately linked to the differentiation status of the host cell keratinocyte and characterization by three distinct phases of replication[15,20]. The HPV replicative cycle begins in the basal layer of the squamous epithelia, where viral particles arrive through micro wounds and there, they complete their life cycle, taking advantage of the differentiation program of the host epithelial cells (Figure 2)[7,9,20].

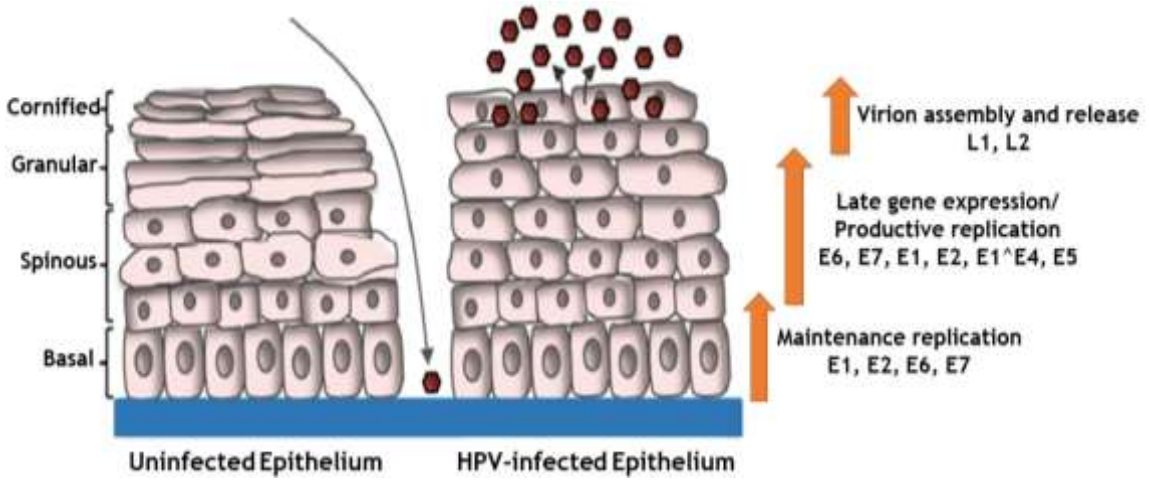

Figure 2. Human papillomavirus life cycle scheme (adapted from Moody, 2017)[20].

After the virus entry, the HPV genome is replicated as an episome[7]. At the initial infection in undifferentiated basal cells, low levels of the E1, E2, E6 and E7 proteins are expressed, delaying normal keratinocyte differentiation[7]. E1 is the only viral protein with enzymatic activity - it is a helicase/ATase which is recruited by E2 at the origin of viral replication, generating approximately 50 to 100 copies of viral episomes per cell[20]. These numbers increase to thousands of DNA viral copies throughout the differentiation of the epithelium[21]. From the middle to upper differentiating epithelial layers, E6 and E7 are expressed in high amounts and the viral life cycle is completed by the expression of L1 and L2 proteins in the uppermost layer of the epithelium[7]. Only upon epithelial differentiation is the productive phase of the viral life cycle activated, resulting in the amplification of viral genomes to thousands of viral copies per cell in the suprabasal layers, as well as activation of late gene expression and virion assembly and release[20]. Regulation of the viral life cycle in this manner allows HPV to avoid detection by the immune response as high levels of viral gene expression as well as virion production are restricted to the uppermost layers of the epithelium, which are not under immune surveillance[20].

Upon reached the basal cells, the L1 protein binds to heparin sulphate proteoglycan (HSPGs)[22], promoting a conformational change in the viral capsid[9]. The conformational change in the capsid exposes the N-terminal region of L2 protein[9], that will be cleaved by the furine, a protein with proteolytic activity, able to interact with different growth factors (such as EGFR, TGF-ß and VEGF), cell and viral receptors[9]. Such actions are needed to promote a second conformational alteration in the viral capsid that allows the virus binding to cell receptors[9]. Next, the virus is internalized in vacuolar structures through endocytic

mechanisms mediated by clathrin and caveolin[9]. Upon lysosomes binding to these endocytic vesicles forming the phagolysosomes, a reduction in pH occurs, leading to the viral capsid disassembling and, consequently, the viral genome release[9,19].

It is important to note that, a complete viral cycle does not represent a risk for cancer development[7], as near 90% of HPV infections, even with HR-HPV, are eliminated within two years[7]. A persistent infection with HR-HPV is the main determinant for cervical cancer development, where the accumulation of damaged DNA, probably due to the interactions of E7 and E6 with their cellular targets, leads to genomic instability and, in most cases, to the integration of the viral genome into the host genome[7]. Viral genome integration precedes the overregulation of E6 and E7 oncogenes, since in most of the cases E2 gene is disrupted, which causes the overexpression of E6 and E7 oncogenes[23].

1.1.4 Oncoproteins E6 and E7

E6 and E7 from HR-HPVs play a key role in carcinogenesis by altering pathways related to the immune response and cellular transformation[12]. The expression of E6 and E7 proteins are associated with the integration of viral DNA into the host genome, malignant transformation and ultimately progression to cancer[6]. These proteins deregulate fundamental cellular events contributing to cancer initiation and progression by altering the cell cycle regulation and telomere maintenance, apoptosis, DNA repair, senescence and differentiation, inducing and facilitating the accumulation of damaged DNA and genomic instability, blocking tumour suppressor pathways and progression towards malignancy[6,12].

Once HPV 16 and 18 are the most frequently detected types in cervical cancer worldwide, their E6 and E7 proteins have been extensively studied[12], lacking enzymatic activities and exert their functions by associating with a broad spectrum of cellular proteins[19]. The E6 and E7 oncoproteins are the primary transforming viral proteins, playing an important role in immune evasion by targeting cytokine expression to alter cell proliferation and interferon responses[6]. If the HPV-infected cells are rapidly eliminated by the immune system, despite the transforming properties of E6 and E7, they do not have sufficient time to accumulate chromosomal abnormalities and acquire a malignant phenotype[12].

However, for the cases that the HPV-infected cells are not eliminated, E6 and E7 constitute attractive therapeutic targets since their inhibition rapidly induces senescence in HPV-positive cancer cells[24]. These viral proteins are the key factors that maintain the malignant phenotype of HPV-positive cancer cells, specifically:

i. E6 and E7 are the only viral genes that are always retained and expressed in HPV-positive cancer cells[24];
ii. both E6 and E7 possess transforming potential in various experimental settings[16];

iii. inhibition of E6/E7 expression results in rapid induction of cellular senescence, an irreversible proliferative arrest, in HPV-positive cancer cells.

The first indication of the oncogenic properties of E6 and E7 was provided by studies on cervical cancer-derived cells, such as SiHa and CaSki cells[12]. In these cell lines, viral DNA was found to be randomly integrated into the host genome, leading to the disruption of several viral genes and preservation of E6 and E7, which were actively transcribed[12]. Many studies have shown that the frequency of viral DNA integration increases with the severity of the cervical lesion, indicating that this event is implicated in the progression of the disease[12].

Several evidences suggest that HPV is involved in the reprogrammed metabolism of cervical cancer cells[7]. Diverse interactions of E6 and E7 with cellular partners have been demonstrated, and many of those affect cell biological functions, leading to transformation[7]. When integrated into the host squamous epithelial cell genome, the high-risk HPV types (16 and 18) will express E6 gene, which will induce the degradation of p53 tumour suppressor protein[6]. E7 also expressed early, is an oncoprotein that binds to the tumour suppressor retinoblastoma protein (pRb) (Figure 3) and allows DNA synthesis[6]. The continuous expression of E6 and E7 are required to maintain the malignant phenotype[7,12,24]. The silencing of both viral genes in cervical cancer-derived cell lines results in rapid cellular death (Figure 3)[12].

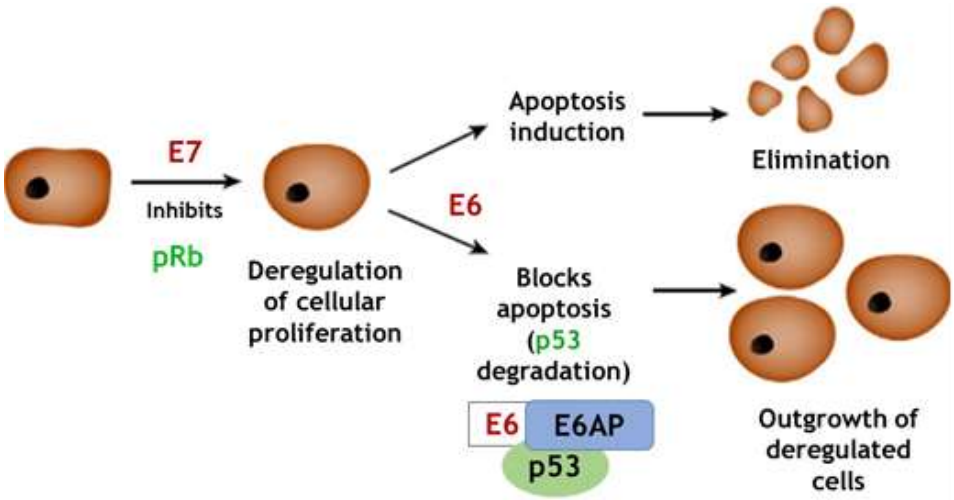

Figure 3. E6 and E7 oncoproteins mechanism of action (adapted from Hope-Seyler and co-workers, 2017)[24].

E6 is a basic and cysteine-rich protein of approximately 150 amino acids and 23 kDa (Figure 4)[12]. This oncoprotein identify some of their target proteins by specifically interacting with acidic LxxLL motifs[25], namely E6AP, the Interferon Regulatory Factor IRF-3 and the Notch co-activator MAML1 through acidic leucine-rich motifs comprising the LxxLL consensus sequence[25]. E6 proteins are rich-cysteine proteins whose key structural characteristic is the presence of two zinc-binding regions, mentioned to as E6N and E6C[12,25], both domains contain two cysteine motifs (CXXC), conserved in the E6 proteins of all HPV types[12]. One of the most

characterized properties of this protein is its ability to induce degradation of the p53 tumour suppressor via the ubiquitin pathway[12], through the interaction of the LxxL motif of E6AP[26]. E6 binds to a short leucine (L)-rich LxxLL consensus sequence of a 100 kDa cellular protein[12] within the cellular ubiquitin ligase E6AP and subsequently the heterodimer E6/E6AP recruits and degrades p53[26]. The E6-associated protein (E6AP), which functions as an E3 ubiquitin protein ligase[12], targeting cellular proteins to be ubiquitinated for proteasomal degradation[7]. The E6/E6AP complex then binds to the central region of p53 which becomes rapidly ubiquitinated and targeted to proteasomes[12]. In this way HR-HPV E6 induces p53 degradation by forming a complex with E6AP[26] and, thus, p53 dependent apoptosis is blocked[7]. E6 interacts not only with LxxL motifs but also with PDZ domains involved in cell polarity and adhesion, and also possesses a self-association interface required for the p53 degradation activity[25]. Neither E6 nor E6AP are separately able to recruit p53, and the precise mode of assembly of E6, E6AP and p53 is unknown[26].

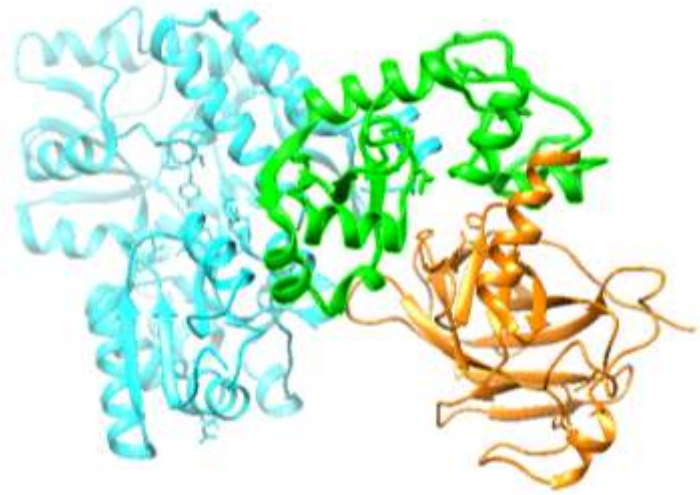

Figure 4. Crystal structure of the HPV16 E6/E6AP/p53 ternary complex (E6AP – blue, p53 – orange, E6 – green) (PDB ID: 4XR8).

E7 is an acidic phosphoprotein of about 100 amino acids and 17 kDa that, likewise to E6, contains zinc-binding motifs being structurally and functionally associated to adenovirus E1A and, grounded on this similarity is divided into three domains: conserved regions 1-3 (CR1-3)[12]. The association of HR-HPV E7 with pRb resulting in the degradation and functional inactivation of pRb[24] via proteasomal pathways, leading to unscheduled cell cycle progression[24], for example with consequent activation of E2F-regulated transcription[12] from their negative control by pRb and, subsequently, the activation of cell cycle-promoting E2F target genes[24]. The genes regulated by E2F1-3 include cyclin A and cyclin E, which are positive regulatory subunits of cyclin-dependent kinase (CDK) complexes whose accumulation results in activation of CDKs and cell cycle progression[12]. The genes regulated by E2F1-3 include cyclin A and cyclin E (both positive regulatory subunit of cyclin-dependent kinase (CDK) complexes), with consequent activation of E2F-regulated transcription[12]. The accumulation of cyclins results in activation of CDKs and cell cycle progression[12].

Furthermore, scientific studies indicate that E6/E7 deregulate the cellular miRNA network, upon repression of endogenous E6/E7 expression in cervical cancer cell lines[26]. Many of these miRNAs have been linked to carcinogenesis, and the expression of several of the most abundant E6/E7-regulated miRNAs is altered in cervical cancer tissues[24]. Vice versa, cellular miRNAs may also be involved in the regulation of HPV E6/E7 gene expression, as it has been reported for miRNA-375[27].

1.1.5 p53 protein

The p53 protein is a stress sensor regulating many cell pathways, such as cell cycle arrest, senescence, apoptosis, metabolic alterations, DNA repair and other mechanisms of tumour suppression[28]. The p53 pro-apoptotic tumour suppressor is mutated or functionally altered in most cancers[26]. This protein consists of a transactivation domain (amino acids 1-44), a proline-rich domain (102-292), a tetramerization domain (325-356) and a C-terminal domain (357-393)[29]. The prevalence (95%) of p53 mutations in human cancers are missense mutations, mostly located within the DNA-binding domain (amino acids 102-292) with hot spots at codons R175, G245, R248, R249, R273 and R282[30].

In the majority of human cancers, p53 activity is often impaired by missense mutations distributed within the exons 4-8 in the TP53 gene, encoding for the DNA-binding domain[31]. In most cases, mutated p53 proteins become inactive but acquire stability and accumulate in cancer cells[31]. Some mutant p53 proteins, besides losing their oncosuppressor activities, acquire oncogenic features (gain of functions) that endow the cells with overgrowing and survival advantages[30].

p53 is a transcription factor that regulates the expression of genes encoding regulators of the cell cycle, DNA repair machinery and apoptosis[12]. Under cellular stress, such as hypoxia or DNA damage, p53 triggers cell cycle arrest or apoptosis to guarantee the integrity of cellular genome[12]. By blocking the cell cycle, p53 prevents the replication of damaged DNA and allows the cell to repair the damage before S phase[12]. Alternatively, if DNA damage is too great and difficult to repair, p53 can divert the cell into apoptosis, thus preventing the production of potentially transformed progeny[12].

In epithelial tumours induced by high risk mucosal human papillomavirus, including human cervical carcinoma and a growing number of head and neck cancers, p53 is degraded by the viral oncoprotein E6[26].

1.1.6 pRb protein

Most cancers manage to impair retinoblastoma protein (pRb) role, whichever through direct mutation of the Rb gene or, more frequently, through the altered expression of their regulators, including cyclin D, CDK4 and CDK6, and their principal inhibitor p16[32].

This protein is composed by 928 amino acids and is usually described as having three domains (Figure 5)[32]. A central domain was recognized as the minimal region necessary to bind viral oncoproteins, such as adenovirus E1A, SV40 Tag and human papillomavirus E7, and it was termed the "pocket", that comprises two subdomains, A and B, each resembling a cyclin fold with three extra helices, cooperating with each other through an extensive non-covalent interface such that the pocket folds into a single structural unit[32]. Two additional cyclin folds constitute the structured amino-terminal domain RBN, which resembles the pocket structure except for a few subtle differences[32]. Roughly the last 150 residues of RB form the carboxyl-terminal domain (RBC), which is inherently disordered[32].

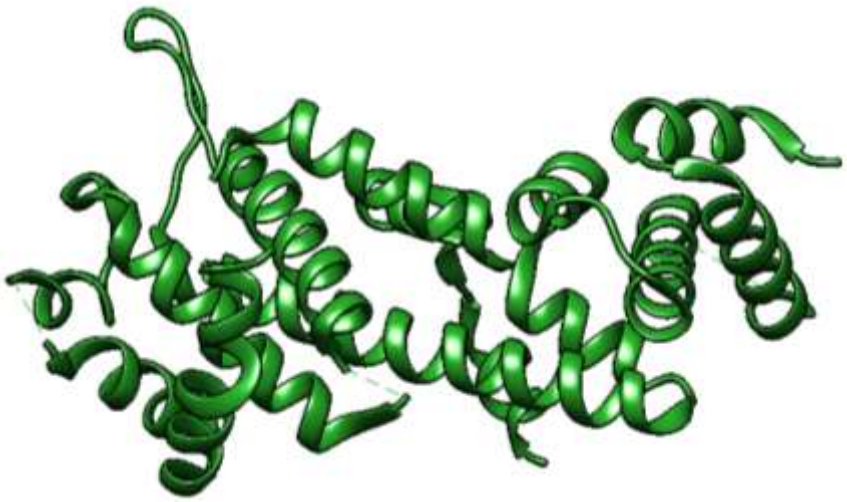

Figure 5. Crystal structure of the pRb (PDB ID: 2QDJ).

Abolishing pRb function permits uncontrolled cell cycle progression and leading to tumour growth promotion[32]. In its simplest form, pRb functions primarily rely on the inactivation of transcription factors, such as E2F responsible for targeting genes in the G1 phase of cell cycle, so that when freely available this protein stimulates cellular cycle transition to S phase[33,34]. Growth factors signal arouse the assemblage and activation of cyclin D and its associated cyclin-dependent kinases (CDK4 or CDK6) that firstly phosphorylate pRb in the G1 phase, which releases the respective E2F family member from pRb, leading to the activation of transcriptional targets to advance the cell cycle[34]. The abundance of mutations affecting members of this pathway, and the resulting lack of proliferative control, has led to the idea that pRb pathway disruption is a hallmark of cancer[34].

Upon viral infection, E7 is expressed and binds to pRb, disrupting its complex with E2F and contributing to continuous cell proliferation[33]. Furthermore, E7 oncoproteins is also implicated with the interference of p107 and p130 functions, two proteins that also regulate cell-cycle proliferation through E2F transcription factor binding[33]. The ultimate inhibition of pRb, p107 and p130 by E7 viral protein contributes to the uncontrolled cell proliferation and progression to malignant transformation seen in HPV-infected cervical epithelium[33].

1.2 Cell apoptosis

Apoptosis or programmed cell death is a regulator of physiological growth control and tissue homeostasis, as well as controlling functions of the immune system being characterized for presenting typical biochemical and morphological hallmarks, in particular, cell shrinking, nuclear DNA fragmentation and membrane blebbing[35,36,37].

Apoptosis usually maintains the integrity of healthy tissues and organs in response to DNA damage, cellular stress or oncogene expression through the eradication of damaged cells, thereby blocking tumour growth[35]. In the recent years, one of the most significant developments in cancer research is the acknowledgement that cell death mostly by apoptosis is significantly tangled in the regulation of tumour formation and also critically determine treatment response[36].

Apoptosis is a tightly controlled and strictly regulated pathway process that eliminates unwanted or potentially harmful cells, once all tumour cells require inhibition of apoptotic pathway to survive, that becomes deregulated in tumorigenesis[35].

There are two basic pathways that lead to apoptosis: the intrinsic mitochondrial pathway and the extrinsic death receptor mediated pathway (Figure 6)[35].

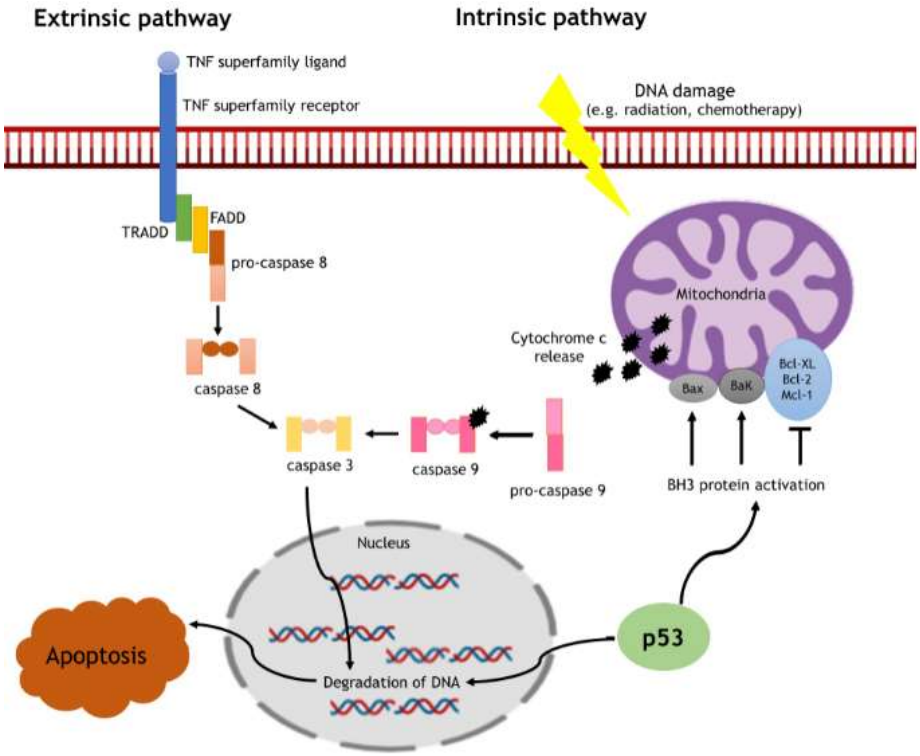

Figure 6. Extrinsic and intrinsic apoptosis pathways schematic representation (adapted from Yuan and co-workers, 2012)[38].

Killing of tumour cells by most anticancer strategies currently used in clinical oncology, for example chemotherapy, γ-irradiation, suicide gene therapy or immunotherapy, has been linked to activation of apoptosis signal transduction pathways in cancer cells such as the intrinsic and/or extrinsic pathway[36].

Proteolytic enzymes such as caspases (a family of cysteine proteases that act as common death effector molecules in several forms of cell death) are important effector molecules in apoptosis, once their activation in response to anticancer therapies can be initiated through activation of the extrinsic (receptor) pathway or at the mitochondria by stimulating the intrinsic pathway[36]. Caspases are synthetized as inactive proforms and upon activation, they cleave next to aspartate residues, resulting in amplification of caspase activity through a protease cascade[36].

1.2.1 Extrinsic pathway

The extrinsic pathway of apoptosis is associated with death receptors that include members of tumour necrosis factor (TNF) superfamily, this is TNF receptor-1, Fas death receptor-3, TRAIL receptor-1 and receptor-2 and death receptor-6, Fas ligand (Fas-L), TNF-related apoptosis inducing ligand (TRAIL) are involved in the extrinsic pathway[35,36]. Members of the TNF receptor family share similar, cysteine-rich extracellular domains[36].

Death receptors consists of more than 20 proteins with a broad range of biological functions, including regulation of cell death and survival, differentiation or immune regulation[36] and are composed by 80 amino acids in their cytoplasmatic domain, denominated the death domain (DD)[35], which plays a crucial role in transmitting the death signal from the cell's surface to intracellular signalling pathway[36].

The DD is essential for the initiation of apoptotic signalling and transduction via transmitting death signal from cell surface to a variety of intracellular targets, where a specific ligand or an agonist antibody can activate the receptors and trigger the apoptotic signalling[35,36].

Trimerization of Fas and TRAIL receptors, the serial recruitment of the adaptor protein Fas-associated DD (FADD), pro-caspase-8, caspase-10 and c-FLIP to Fas DD could ultimately form the death-inducing signalling complex (DISC), culminating in the activation of the caspase-8 and the cell is committed to apoptosis[35]. Though, in addition to FADD, the TNRF1-associated death domain (TRADD) is involved in formation of DISC in the case of TNF-R1 activation, initiating a cascade of events, leading to a caspase-8 activation and lastly to apoptosis signalling[35].

1.2.2 Intrinsic pathway

In the mitochondrial pathway of apoptosis, caspase activation is closely linked to permeabilization of the outer mitochondrial membrane by proapoptotic members of the Bcl family[36], once internal stimuli (include growth factor deprivation and oxidants[35]) promote the activation of caspases and pro-apototic members of the Bcl-2 family, leading to mitochondrion outer membrane permeabilization (MOMP) via Bax channels[37].

Numerous cytotoxic stimuli and proapoptotic signal-transducing molecules converge on mitochondria to induce outer mitochondrial membrane permeabilization[36]. Internal stimuli promote the activation of caspases and pro-apototic members of the Bcl-2 family, leading to mitochondrion outer membrane permeabilization (MOMP) via Bax channels[37]. This permeabilization is regulated by proteins that regulate bioenergetic metabolite flux and components of the permeability transition pore[36]. Upon disruption of the outer membrane, a set of proteins normally found in the space between the inner and outer mitochondrial membranes is released, including cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G, that once in the cytosol, these apoptogenic proteins trigger the execution of cell death by promoting caspase activation or by acting as caspase-independent death effectors[36]. In its turn, MOMP leads to the release of cytochrome-C, which induces the formation of the apoptosome, a complex seven caspase-9 units[37]. Caspase-9 will trigger an executioner caspase (3 or 7) ensuring the continuity of the process[37]. The anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL, Mcl-1) are able to inhibit apoptosis by quenching the activity of their pro-apoptotic counterparts[37].

1.3 DNA based therapy

The use of DNA biopharmaceuticals is becoming very attractive for biological therapy due to the fact that the therapeutic transgenes of interest are generated by the cell transcriptional machinery[39]. In this way, much attention has been drawn recently for new forms of immunotherapy for infectious diseases and cancer. DNA vaccines still possess the intrinsic advantage of target flexibility, low cost, and physical stability over a wide range conditions that may be encountered when targeting diseases with global distribution[40]. Similar to other gene-based therapies, this technology plays a key role in the final efficacy of DNA vaccines[40].

1.3.1 DNA vaccines

DNA vaccines have been considered as the third generation of vaccines, promising humoral and cellular immune responses, presenting some advantages such as low cost production, thermostability and easy distribution when comparing to the conventional ones[33]. The immunological technology is based on the delivery of genetic information aiming to induce an immune response against a specific antigen (chosen to be encoded in the DNA vaccine must

be related with a given pathology)[33]. Subsequently inoculation, the treated individual should be capable of producing a strong immune response against the antigen for which genetic information was beforehand delivered, triggered mainly as a result of the transfection of antigen-presenting cells (APCs) and non-APCs that will later express the antigen encoded in the DNA vaccine[33].

Due to the reason that DNA vaccines can have preventive and therapeutic effects, they are currently under research in the treatment of cancer, once numerous cancers show specific antigens that could be used in the development of a DNA vaccine to induce a specific immune response against those antigens[33].

Researchers have mostly considered that the majority of DNA vaccines developed to the date do not present the high-level immunogenicity observed in mouse models, therefore mitigating the lack of DNA vaccines approved for human use, being the reason for new methodologies be currently under study to improve the clinical outcome of DNA vaccines, appearing from the remarkable progress that has been observed in the past years for this area of studies[33].

1.3.2 Gene therapy

Gene therapy is a promising strategy to treat acquired diseases and/or genetic disorders, aiming to deliver genetic material into target cells or tissue and to express it with the intention to gain a therapeutic effect[1], thus regulating cellular processes and responses[41]. Relying on the purpose and delivery method, successfully delivered genes could have several destinies: modifying defective host genes, replacing the deficient host genes, inserting into the host genome or staying in the nucleus without integration into the host genome[41].

The European Medicines Agency (EMA) defines that a gene therapy medicinal product is a biological therapeutic product which fulfills the following two characteristics: (a) contains an active substance which consists of a recombinant nucleic acid used in or administered to human beings with a view of regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence. Gene therapy medicinal products shall not include vaccines against infectious diseases.

Gene therapy has the advantage over traditional therapies since it can be administered locally, allowing the delivery of a high therapeutic dose without taking the risk of rising systemic adverse effects[1]. The subsequent transgene expression can re-establish normal cellular processes or induce new cellular responses, therefore realize the aim of therapy[41]. However, the main challenge in gene therapy remains in being able to deliver an adequate amount of genetic material into target cells or tissues and to maintain gene expression for a

desired period of time[1]. In addition, since most gene therapies are of single time application, in the long run, they can be cost-effective[1].

The perfect delivery system should be capable of ensuring a successful gene therapy and must satisfy the following criteria[42]:

i. must not interact with vascular endothelial cells and blood components;
ii. be required to be capable of avoiding uptake by the reticuloendothelial system;
iii. ought to be small enough to pass through the cell membrane and reach the nucleus.

Generally, gene therapy can be categorized into two categories: germline and somatic gene therapy[1]. The difference between these two approaches is that in somatic gene therapy genetic material is introduced in some target cells, but the change is not passed to the next generation, while in germline gene therapy the therapeutic or modified gene will be passed to the next generation[1]. This is a major and very important difference since current legislation only allows gene therapy on somatic cells[1].

To improve gene transfer efficiency, specificity, and safety, numerous gene transfer systems, usually called gene vectors have been developed, being divided into two categories: viral and non-viral gene delivery[41].

1.3.2.1 Gene therapy for cervical cancer

Since the effects of E6 and E7 are the loss of function of p53 and pRb, respectively, it seem likely that HPV-associated tumours could be controlled by replacement of wild-type p53 or pRb proteins in a functional form[43]. The potential use of p53 or pRb as gene therapy has been examined for many types of cancers, quite apart from those that are associated with HPV and has progressed to the stage of multiple human trials[43].

In 2012, several clinical assays were in progress using several DNA plasmids, summarized in Table 1[44].

Once the HR-HPV principal tumorigenic effects have been related to the expression of E6 and E7 genes, a remarkable effort have been done to develop a suitable gene therapy for blocking their expression[43]. The first method for blocking the translation of the RNA to protein was the use of an antisense RNA, as this molecules can bind to the RNA of the opposite strand, leading to a duplex, that soon will be degraded, resulting in a reduction of the gene expression[43]. Studies using the original HPV antisense constructs were expressed from plasmids containing E6 and E7 genes in the opposite orientation to the virus in relation to their promoter and were tested in C4-1 cervical cancer cell line, showing a loss of several features of transformed cells and also established practicability of blocking expression and therefore eliminating the malignant phenotype[43].

Other approaches used for the prevention of gene expression have been exploited [43]. For instance, the E6 protein may have its function blocked by RNA molecules that bind specifically, known as an aptamer, having an investigation group showed that this could cause apoptosis in HPV-16-positive cancer cells but not HPV-negative cells[43].

Table 1. Clinical assays approved for cervical cancer gene therapy (using a naked plasmid DNA as vector)[43].

ID trial	Title	Country	Clinical phase	Genes transferred
US-1093	Phase III placebo-controlled study of VGX-3100, (HPV 16 E6/E7, HPV 18 E6/E7 DNA vaccine) delivered intramuscular followed by electroporation.	USA	Phase II	HPV 16 E6/E7 fusion protein HPV 18 E6/E7 fusion protein
US-0595	pNGVL4a-Sig/E7/HSP70 for the treatment of patients with HPV 16 cervical intraepithelial neoplasia 2/3	USA	Phase I and II	HPV 16 E7 oncogene
US-0916	Dose escalation study to evaluate the safety, tolerability and immunogenicity of HPV DNA plasmid (VGX-3100) + electroporation in adult females with histological diagnosis of grade 2 and 3 cervical intraepithelial neoplasia	USA	Phase I	HPV 16 E6/E7 oncogenes HPV 18 E6/E7 oncogenes
US-0984	pNGV4a-CRT/E7 for the treatment of patients with HPV 16 cervical intraepithelial neoplasia 2/3	USA	Phase I and II	HPV 16 E7 oncogene
US-1040	Evaluate the safety, tolerability and immunogenicity of a 4th dose of HPV DNA plasmid (VGX-3100) + electroporation in adult females previously immunized with VGX-3100	USA	Phase I	HPV 16 E6/E7 oncogenes HPV 18 E6/E7 oncogenes

1.3.3 DNA vectors

An ideal vector should satisfy several criteria[42]:

i. must not cause a strong immune response;
ii. should be capable of transporting nucleic acids regardless of their size;
iii. need to lead to a sustained and regular expression of its genetic cargo;
iv. ought to deliver the gene to only certain type of cells, especially when the target cells are scattered throughout the body, or when they are part of a heterogeneous population;
v. be required to be able to infect both dividing and non-dividing cells;

vi. must be of easy preparation, inexpensive and commercially available at high concentrations;
vii. need to either remain in episomal position or integrate into a specific region of the genome, but not integrate randomly.

In fact, viruses (Table 2) were the first carriers to be used to deliver and protect the therapeutic gene, benefiting from the virus life cycle[41,42]. This type of carrier, known as a viral vector, is one of the most used vectors in gene therapy, also due to its capacity to carry the gene efficiently and guarantee long term expression[41,42]. Though, the risk of a immune response caused by the use viruses as delivering vectors, its high cost and difficulty relating to their preparation and the limited size of genetic materials that can be inserted into human cells, as well as the associated immunogenicity have constrained the use of these vectors in gene therapy and led to research into safer and cheaper and safer alternatives[41,42].

Therefore, non-viral vectors have appeared, presenting several advantages that include relatively safety, generally causes low immune response, can be easily prepared at low cost and in large quantities, have the ability to transfer large size genes, cause less toxicity and can be modified with ligands for tissue or cell-specific targeting[41,42]. In addition, they can transfer different and large transgenes, being able to be stored for long periods due to their stability[42]. However, non-viral methods present limitations of low transfection efficiency and poor transgene expression[41].

The non-viral approaches can be divided into two groups (Table 3): physical approaches and chemical vectors[42]. The physical approaches rely on a physical force that weakens the cell membrane to facilitate the entrance of the gene into the nucleus and include needle injection, electroporation, gene gun and ultrasound[42]. The chemical vectors can be prepared by electrostatic interaction between a polycationic derivatives, which can be lipids or polymers, and the anionic phosphate groups of the DNA to form a particle called polyplex. On the other hand, a lipoplexe is formed when the DNA interacts with a lipid or by encapsulation of DNA within biodegradable spherical structures that lead to micro or nanoparticles containing DNA, or by adsorption of DNA[42].

Table 2. Viral vectors and its major advantages and disadvantages (adapted from Ibraheem and co-workers, 2014)[44].

Vector	Advantages	Disadvantages
Adenovirus	Very high titers (10^{12} pfu/mL) High transduction efficiency Transduces proliferating and nonproliferating cells Production easy at high titers	Remains episomal Transient expression Requires packaging cell line Immune-related toxicity with repeated administration Potential replication competence No targeting Limited insert size: 4-5 kb
Adeno-associated virus	Integration on human chromosome 19 (wild-type only) to establish latent infection Prolonged expression Transduction does not require cell division Small genome, no viral genes	Not well characterized No targeting Requires packaging cell line Potential insertional mutagenesis High titers (10^{10} pfu/mL) but difficult production Limited insert size: 5kb
Herpes simplex virus	Large insert size: 40-50 kb Neuronal tropism Latency expression Efficient transduction *in vivo* Replicative vectors available	Cytotoxic No targeting Requires packaging cell line Transient expression, does not integrate into the genome Moderate titers (10^{4}-10^{8} pfu/mL)
Lentivirus	Transduces proliferating and nonproliferating cells Transduces hematopoietic stem cells Prolonged expression Relatively high titers (10^{6}-10^{7} pfu/mL)	Safety concerns: from human immunodeficiency virus origin Difficult to manufacture and store Limited insert size: 8 kb Clinical experience limited
Retrovirus	Integration into cellular genome Broad cell tropism Prolonged stable expression Requires cell division for transduction Relatively high titers (10^{6}-10^{7} pfu/mL) Larger insert size: 9-12 kb	Inefficient transduction Insertional mutagenesis Requires cell division for transfection Requires packaging cell line No targeting Potential replication competence

Table 3. Non-viral vectors delivery methods (adapted from Ibraheem and co-workers)[44].

Physical methods	Electroporation	Induce the uptake of injected DNA into the cell, by increasing the permeability of the cell membrane through exposure to a controlled electric field. Applied to several tissues, such as skin, muscle, liver, and tumour. Efficacy is affected by the pulse duration, field intensity, cell size, shape, and density. Safe, efficient and reproducible. Difficult to employ for transferring DNA into a large area of tissue requires surgery to install the electrode in the internal organs and can cause incurable harm and mutilation in the tissue treated due to the high voltage applied.
	Gene gun	The transgene delivery into the target cell/tissue is carried out by the use of accelerated particle carriers' biocompatible heavy metals (gold, tungsten, silver). The efficiency is determined by the size of the particles employed as DNA-carriers, gas pressure used to accelerate them, and the dosage of transgene blasted into the target. Effectively employed in genetic vaccination, immune therapy and suicide gene therapy to treat cancer. Presents high-level gene expression quickly achieved, long-lasting gene expression and can reach numerous organs without injury the surrounding tissues. Shows a low efficiency when used to transfer the gene onto whole tissue due to low penetration by the metal particles and often needs surgery to apply the approach for deep tissues.
	Ultrasound	Depends on increasing the permeability of the cell membrane by using ultrasound waves to create pores or acoustic cavitation in the exposed cell membrane. It is safe, non-invasive and it can reach internal organs without the need for surgery. The efficiency depends on the frequency, intensity and period of cell exposure to the ultrasound irradiation, DNA concentration, the use of a contrast factor.
Chemical vectors	Electrostatic interaction	This system uses the electrostatic attraction between the anionic DNA and a cationic lipid or polymer, leading to a positive complex know as lipoplex or polyplex, respectively. The major problem hindering the use of lipoplex *in vivo*, besides poor efficiency, is the cytotoxicity resulting from the lipoplex positive charge. Moreover, the use of polyplexes *in vivo* must overcome many obstacles, such as their toxicity, poor efficiency, and polymer polydispersity.
	DNA encapsulation	Many strategies have been developed and conducted to deliver nucleic acids for gene therapy application. DNA can be transported using various kinds of organic and inorganic particles such as silica nanoparticles, carbon nanotubes, and functionalized

		superparamagnetic iron oxide nanoparticles. Liposomes are structures formed spontaneously when certain lipids are placed in the aqueous phase, through the self-assembly of these lipids in such a way as to orient their hydrophobic parts away from the water, while the hydrophilic parts are oriented towards the aqueous phase surrounding the hydrophobic ones.
	DNA adsorption	This technique is the result of combining electrostatic interaction and encapsulation, where the positively charged polymers adsorb on the surface of biodegradable particles to which DNA can be electrostatically linked. Improves DNA bioavailability and augment loading efficiency.

1.4 Micro RNA (miRNA)

miRNA are a class of endogenous, single-strands non-coding small RNAs with 17-25 nucleotides that are involved in regulating gene expression at the post-transcriptional level [10,45,46].

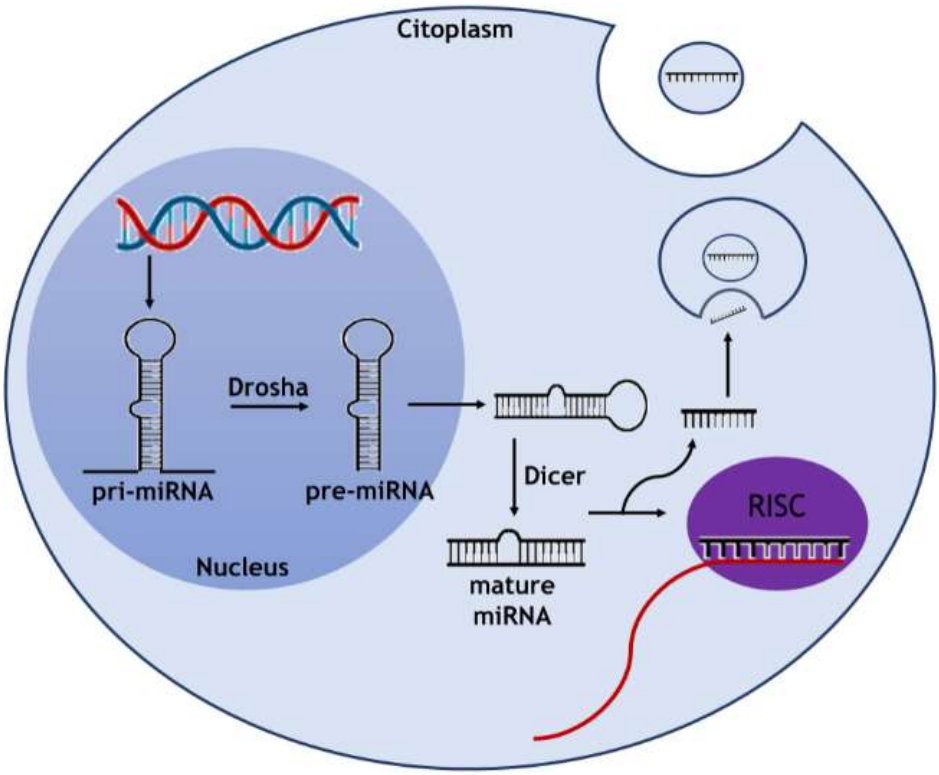

Figure 7. The biogenesis of miRNA scheme (adapted from Etheridge and co-workers, 2011)[47].

Long primary transcripts of miRNA (pri-miRNAs) are mainly transcribed by RNA polymerase II and partially by RNA polymerase III within and outside the nucleus by Drosha and Dicer, respectively[48,49]. The pri-miRNA is hundreds of nucleotides long and modified by adding a 5' cap and a 3' poly-A tail[49]. These pri-miRNAs are processed by a RNase III endonuclease Drosha into hairpin precursor miRNAs (pre-miRNAs) that are about 70 nucleotides long and are exported from the nucleus to the cytoplasm[46,49]. The pre-miRNAs are subsequently processed

by another RNase III endonuclease called Dicer to form a mature miRNA duplex with 17-25 nucleotides[49]. One of the strands (guide strand) of the mature miRNA is incorporated into a miRNA induced silencing complex that is composed of the Argonaute (AGO) protein family and the transactivation-responsive RNA binding proteins[49]. miRNAs mediate the target mRNA repression or degradation via partial or complete base pairing to the 3' untranslated region of the target mRNA[49]. The other strand (passenger strand) of pre-miRNA is either degraded or exported from cells by exosomes, microvesicles, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) or RNA binding proteins and then released into the circulation (Figure 7)[47].

The mature miRNAs function by incorporating into RNA-induced complementary sites in the 3' untranslated region (3'UTRs) of messenger RNAs (mRNAs), which causes translation repression and, in some cases, the degradation of the target mRNA[48]. These small nucleic acids (miRNAs) can be applied to discover unknown cancer origins, identify therapeutic targets and classify cancer subtypes, as well as being correlated with tumorigenesis, cancer progression, prognosis and treatment response[50,51]. Depending upon the function of the gene product of miRNA target, the tissue type, mRNA target and microenvironment[49], the end result of any miRNA action could be tumour promoting (oncomirs) or tumour suppressor[51]. In addition, miRNA dysregulation is involved in many critical pathways in cancer development such as those that regulate apoptosis, cell proliferation, epithelial-to-mesenchymal transition and metastasis[49].

1.4.1 miRNA-375

Human miRNA-375 is located on chromosome 2 between CRYBA2 and CCDC108 genes and is transcribed from its own promoter[48]. The region upstream of the pre-miRNA-375 sequence is found highly conserved, implicating the possibility of these sequences for transcription factors binding and gene expression control[48]. Many studies have found that miRNA-375 upstream sequence contains transcription factors binding sites such as TATA box and E-box[48]. TATA box has the core DNA sequence 5'-TATAAA-3' to which transcription factors bind and get involved in the process of transcription by RNA polymerase, while E-box contains a specific DNA sequence, typically CACGTC, which can be recognized and bound by transcription factors usually containing the basic helix-loop-helix (bHLH) motif and mediates transcription initiation[48].

A recently described mechanism that potentially contributes to the activation of E6 and E7 is the downregulation of miRNA-375 in multiple types of cancer and acts as a tumour suppressor by inhibiting malignant properties of cancer cells[27,48]. The expression of this miRNA was shown to be repressed by methylation of CpGs located in its promotor region[52]. Enhanced methylation of this region could be observed in transforming HPV-infections including preneoplastic lesions as well as invasive carcinomas[53]. Consequently, expression of miRNA-375

decreases during HPV-mediated cervical transformation[52]. Interestingly, miRNA-375 was shown to suppress the expression of multiple host cellular and viral oncogenic factors including the transcription factor SP1, the E6 associated protein (E6AP) and the E6 and E7 oncogenes of HPV 16 and 18[27]. Thereby, miRNA-375 may contribute to the intracellular surveillance to prevent the oncogenic activity and is therefore suggested to play a tumour suppressive role, especially in HPV-associated cancers[48].

The alteration of miRNA-375 in cancer can be caused by a variety of mechanisms, including the dysregulation of transcription factors and aberrant promotor methylation[48]. Reduced expression of miRNA-75 in tissue or circulation may indicate the presence of neoplasia as well as poor prognosis of many malignant cancer[48].

1.5 Minicircular DNA (mcDNA)

mcDNA is a new biotechnological product with beneficial therapeutic perspectives for gene therapy as it is exclusively constituted by the eukaryotic transcription unit, which improves mcDNA safety and increase of its therapeutic effect[54,55]. The absence of prokaryotic sequences in this vector, namely, the antibiotic resistance region and the origin of replication, improves its biocompatibility and efficacy in comparison with plasmid DNA (pDNA)[56]. In addition, these molecules are small and more stable than pDNA, which increases the bioavailability[57], allows the improvement of the transfection efficiency and biological activity[58]. mcDNA is defined as an enabling technology for gene transfer into mammalian cells and is claimed to be comprised of a unique array of characteristics that include[39]:

i. inherent supercoiled topological isoform;
ii. lack of any bac-ORI;
iii. absence of any sequence coding for antibiotic resistance;
iv. existence of a therapeutic transgene whose expression is controlled by a mammalian promoter;
v. existence of specific sequences attributed to the sites of recombination where the precursor parental plasmid (PP) yields the minicircle.

In addition, due to the absence of CpG motifs, the gene will be expressed over several weeks, reducing the death of transfected cells, lying another advantage in obtaining higher therapeutic expression levels[59].

The widespread use of clinical-grade mcDNA biopharmaceuticals is remarkably challenging from a manufacturing point of view since the conversion of template PP into mcDNA is a relatively complex process[39]. Prior to the induction stage, mcDNA precursor plasmids are generally amplified in gram-negative *E. coli* bacteria during fermentation because this recombinant organism presents unique characteristics including fast growth kinetics, high

population density and high ratio of growth/nutrient consumption[60]. Following PP amplification, the induction stage of the PP-mcDNA conversion is stimulated by the addition of an inductor to activate the expression of genes encoding for the recombination machinery[39]. This genes activation will promote the expression of the cellular machinery responsible for the conversion of PP templates into mcDNA, followed by the precise action of site-specific recombinases that start minicircle biosynthesis (recombination)[39]. Throughout this dynamic production process, different species are generated: the mcDNA of interest, as well as residual plasmids containing bacterial elements such as PP precursors and mP[39].

1.5.1 Production

As it is schematized in Figure 8, the production of this biomolecule results from the intramolecular recombination of the parental plasmid (PP) in a bacterial culture[56] induced by L-arabinose which provides phuC31 serine recombinase expression, resulting in the formation of mcDNA (containing the therapeutic gene) and of mini plasmid (mP, containing the bacterial elements)[54]. The simultaneous expression of I-SceI endonuclease promotes the digestion of PP and mP[57].

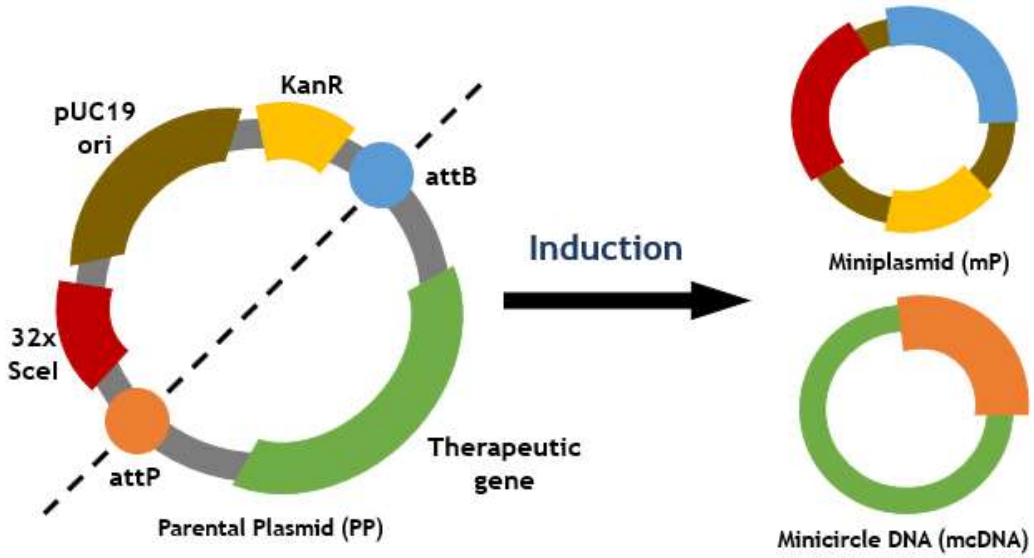

Figure 8. mcDNA production scheme from parental plasmid (adapted from Gaspar and co-workers, 2014)[55].

1.6 Purification

The purification of biopharmaceuticals is commonly considered the bottleneck of the manufacturing process[61]. Increasing product diversity, along with growing regulatory and economic constraints raise the need to adopt new rational systematic and generally applicable purification processes[61].

The purification of a biopharmaceutical is not a simple task[61]. Besides the cell debris, host cell proteins, genomic DNA, endotoxins and RNA all together should be removed. In addition,

product-related impurities such as product moieties that are misfolded, aggregated, carry the wrong post-translational modifications, loss the bioactive conformation or are otherwise chemically degraded may complicate the purification step, due to their structural similarity to the target molecule[61].

1.6.1 Chromatography

Biopharmaceuticals have been a major driving force in the pharmaceutical industry in recent years[61]. Despite increasing competition from non-chromatographic techniques, and pressure to reduce costs and increase throughput, packed-bed chromatography is still the dominant technique in biopharmaceutical isolation stage[61]. This prevalence is mainly due to the high-resolution purification that can be achieved even for similar components61. Thus, liquid chromatography is considered the key step in most purification processes and well-understood unit operation[61].

Liquid chromatography is one of the most widely used methods in biotechnology both at analytical, preparative and industrial level[62]. Due to its simplicity, robustness, versatility and high reproducibility, it can be applied in the purification of a variety of biomolecules[62]. Nowadays, for therapeutic areas under development, such as gene therapy and DNA vaccination, the major interest is not only to produce high quantities of plasmids as non-viral vectors but also to obtain and maintain their quality, meeting the purity requirements of regulatory agencies[62].

Different chromatographic techniques, including size exclusion, anion exchange, hydrophobic interaction, reverse phase or affinity chromatography have been continuously developed and implemented to isolate the biotarget from the remaining host impurities[62]. Nevertheless, liquid chromatography is certainly the most extensively implemented technique[63].

Many chromatographic methods are applied solely or mixed as purification techniques of nucleic acids[64]. The chromatographic purification step usually faces plentiful challenges due to the physiochemical properties resemblances like size, hydrophobicity, and charge, between the nucleic acid and other lysate components, which may complicate the separation process[64].

1.6.1.1 Hydrophobic interaction chromatography

The hydrophobic interaction chromatography (HIC) exploits the interaction between hydrophobic regions on the surface of biomolecules and hydrophobic ligands immobilized on the supports[65]. Due to the variances in the hydrophobicity of the bacterial lysates, HIC raised great attention as a purification tool of pDNA[64,65].

The retention in an HIC happens due to hydrophobic interactions among the hydrophobic ligands on the stationary phase and the apolar moieties on the macromolecular surface in the presence of a salt[65]. It is important to highlight that the concentration of salt in the mobile phase buffer that is passing through the hydrophobic support affects the separation of pDNA, once high salt concentrations improves the interaction between the pDNA and the hydrophobic support[64]. HIC separations are performed in four steps[64]:

i. Equilibration: preparation of the stationary phase by passing a buffer containing salt (generally sodium phosphate or ammonium sulphate);
ii. Sample application and wash step: introduce the sample into the stationary phase and wash the weakly bound impurities using moderate concentration of the salt;
iii. Elution: performed using a gradient decrease of the salt concentration in order to separate the pDNA isoforms.
iv. Regeneration: removing all the strongly bound molecules, using for example organic solvents.

1.6.1.2 Ion exchange chromatography

Ion exchange chromatography (IEX) is a chromatographic technique that separates ions and polar molecules, therefore prior to performing an IEX, it is important to assure that electroneutrality of the stationary phase is achieved[66]. The main types of IEX are anion and cation exchange[66].

In cation-exchange chromatography (CEC), the stationary phase is negatively charged; hence, it can separate cations form other ions[66]. On the other hand, in anion exchange chromatography (AEC), the stationary phase is positively charged and can separate anions form other ions[66].

The AEC is employed in the purification of pDNA using positively charged stationary phases, taking advantage of the negatively charged phosphate groups of pDNA[64]. Although the lysate contains different biomolecule topologies which may have similar overall charge and molecular weight, the differences of the conformations change the distribution of the charge densities[64]. Therefore, by elevating the salt concentration, the pDNA isoforms will be separated according to the increasing order of charge density, eluting first the oc pDNA, followed by the linear pDNA and finally the sc pDNA[64].

1.6.1.3 Affinity chromatography

Affinity chromatography (AC) is a form of chromatography, usually applied for purification of biological mixes depending on highly selective reversible interactions between a ligand loaded onto a support and the substrate[64]. Normally, the more interaction sites between the ligand and the substrate are, the higher dynamic binding capacity of the ligand will be, which

might contribute to the whole technique selectivity and versatility of affinity chromatography, nominate it to be a meaningful tool for purification of several biological molecules like pDNA, RNA, DNA and proteins[67].

The purification of pDNA from lysates is grounded on the fact that pDNA binds to affinity ligands through different styles of bonds and interactions like hydrogen bonds, ionic bonds, hydrophobic interaction, etc, while the other lysate components (impurities) like RNA, gDNA, proteins and endotoxins, employ different affinities to the ligands at certain mobile phase's salt concentration, pH and temperature[64]. Therefore, the separation of the pDNA isoforms happens as the impurities were washed out while the pDNA molecules were retained or vice versa[64].

1.6.1.4 Size exclusion chromatography

Size exclusion chromatography (SEC) separates a mixture of substances with different molar masses and provides a way to purify macromolecules, such as antibodies, proteins, peptides, nucleic acids and industrial polymers with high molecular weight[68] and even nanoparticles[66]. The SEC column is packed with fine, porous polymeric beads, being the size of the pore determinant for the dimensions of the molecules that can be retarded, once if the target molecules are bigger than the size pores, they will be able to pass through the spaces among the packing material running a fast and direct way[66]. On the contrarily, if the molecules are smaller than the pore sizes, they will enter the pore and are retarded by running a long way then the larger molecules[66]. Thus, the first fraction obtained has a higher molecular weight than ones that follow[66].

As described in the handbook "Size Exclusion Chromatography: Principles and Methods" available through GE Healthcare Life Sciences[69], unlike IEX or AC, molecules in SEC do not bind to the chromatography medium so that the buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of size exclusion is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation. As such, this type of chromatography is well suited for biomolecules sensitive to changes in pH, temperature, etc. Besides that, as the samples are eluted isocratically, there is no need for the use of different buffers throughout the separation assay, allowing to maintain the biomolecule stable.

1.7 Applications

After the use of the suitable purification technique in order to achieve a purity degree of the biopharmaceuticals according to the regulatory agencies' guidelines, it is possible to perform transfection studies so that the anticipated therapeutic effect can be evaluated.

Furthermore, especially for the cases where it is expected an improvement of the level of a determined tumour suppressor to induce target cells apoptosis or for those where it is expected to occur the silencing of the oncoproteins responsible for the development of a certain disease, it is imperative to make a rigorous evaluation of the expression level of certain proteins in the transfected cells with the biopharmaceutical of interest. For such, it is possible to use several technologies like Western-Blot, Two-dimensional polyacrylamide gel electrophoresis, Maldi Tof Tof and Orbitrap.

1.7.1 Western-Blot

The Western-Blot is a common method used to detect and determine the size of specific proteins[70] as well as post-translational modifications on proteins and can provide semi-quantitative or quantitative data about the target protein in simple or complex biological samples[71].

Western blotting, also referred to as immunoblot, is an extensively used technique for protein analysis, being a multistep procedure that typically involves the following steps[70,71,72]: (i) sample preparation, (ii) lysis buffer, (iii) determination of protein concentration, (iv) gel electrophoresis, (v) loading gels, (vi) transfer of proteins, (vii) blocking and antibodies incubation, (viii) detection. Very briefly, in this technique, a gel electrophoresis used to separate native or denatured proteins, being then transferred to a membrane and subsequent detection of a target protein by an antibody specific to the target protein[70,73].

A major concern of western blotting is the lack of any consensus on what constitutes reproducibility[71]. Although western blotting seems to be a straight forward and simple method for protein analysis in practice, it is an error-prone method due to its time consuming multistep protocol[72]. Detection of low abundant proteins and post-translational modified proteins such as phosphorylation, acetylation, and methylation are difficult due to the requirement of relatively large sample amounts (usually > 10 µg), as well as high quality validated antibodies against phosphorylated antigens used for western blotting[72].

The presence of proteins may be also evaluated by complementary techniques, such as:

i. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): separates protein according to its isoelectric point (pI) in the first dimension and according to its molecular weight in the second dimension[74];

ii. Maldi Tof Tof: identifies proteins based on the accurate mass measurement of a mixture of peptides derived from a protein[75];

iii. Orbitrap: wide range mass analyser, where sample is injected and protein identification is performed based on m/z ions ratio[76].

Chapter II - Aim

A persistent infection with human papillomavirus (HPV) is the main risk factor for the development of cervical cancer. This cancer is the 4th most common among women worldwide. Up to date, there is no treatment specially development to treat this cancer, urging the need to find new therapeutic strategies. The HPV oncoproteins E6 and E7 are responsible for the tumour progression due to the inactivation of the tumour suppressor proteins p53 and pRb, respectively. In this scenario, the use of biopharmaceuticals that silence the E6 and E7 oncoproteins expression and re-establish the p53 and pRb levels, can be a promising strategy for the cervical cancer treatment. Some success cases of employment of non-viral pDNA vector in several diseases, including cancer, have been reported. However, the possibility to use a safer vector, due to the absence of bacterial genes, that increases the transfection efficiency and persistent expression of the therapeutic gene, turns the mcDNA vector a promising strategy in comparison to the pDNA.

Thus, this dissertation can be divided into the following goals:

- Produce and purify different mcDNA encoding for the primiR-375, p53 and primiR-375+p53 to re-establish tumour protein levels in cervical cancer cell lines;
- *in vitro* transfection studies in cervical cancer cell lines to evaluate the transfection efficiency of the mcDNA-primiR-375, mcDNA-p53 and mcDNA-primiR-375+p53 vectors and the therapeutic effect of the codified gene;
- Evaluate if the mcDNA encoding for both gene presents a higher therapeutic effect in comparison to the mcDNA encoding for the isolated genes.

Chapter III – Materials and Methods

3.1 Production

3.1.1 Bacterial growth conditions

Firstly, in the day prior to the fermentation, *E. coli* ZYCY10P3S2T strain transformed with PP-empty, PP-p53, PP-primiR-375 and PP-primiR-375+p53 were inoculated in LB-agar plates supplemented with kanamycin in the concentration of 50 µg/mL (Thermo Fisher Scientific, United States of America). After growing in a solid medium, the colonies were inoculated into a TB medium pre-fermentation (V = 62.5 mL) with a composition of 20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4, supplemented with kanamycin (50 µg/mL). The bacterial growth was promoted at 42 ºC, under the constant agitation of 250 rpm (Agitorb 200 IC, Aralab, Portugal) until an optical density at 600 nm of approximately 2.6 be achieved. Up next, the amount of bacterial culture needed to transfer to a fermentation (V = 250 mL) was calculated in order to start with an optical density of 0.2. The fermentation started at 42ºC and 250 rpm, until an optical density of approximately 5 be reached. The final step to obtaining the mcDNA was the induction stage, where an induction mixture was added (V = 250 mL) constituted by 25 g/L of LB medium (BIOKAR Diagnostics, France) and 0.04 M of NaOH, L-arabinose for a final concentration of 0.01% (m/V). This induction stage was performed during 3 hours under 250 rpm agitation. Lastly, the cells were recovered through centrifugation at 4500 rpm, during 10 minutes at 4 ºC. The pellets collected were stored at -20 ºC for posterior mcDNA extraction.

3.2 Extraction

3.2.1 Kit Qiagen®

The first approach to obtain purified mcDNA samples was to use QIAGEN® Plasmid Purification kit (QIAGEN®, Germany). For such, the bacteria pellet recovered after fermentation (equivalent to a volume of 250 mL) was resuspended in 20 mL of solution I (50 mM Tris pH 8.0, 10 mM EDTA, 100 µg/mL RNase A). The cell lysis was performed by the addition of 20 mL of solution II (200 mM NaOH, 1% SDS (w/v)) and incubated 5 minutes at room temperature. To neutralize this reaction, 20 mL of solution III (3 M potassium acetate pH 5.5) was added and incubated on ice for 20 minutes. The elimination of cell debris was achieved through two centrifugation steps at 20 000g, during 35 minutes at 4 ºC, in the Allegra™ 25R centrifuge (Beckman Coulter, United States of America), recovering the supernatant between

centrifugations. The supernatant was then added to chromatographic anion exchange columns, previously equilibrated with 10 mL of equilibrium solution (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (V/V), 0.15% Triton X-100) where the molecules of interest were selectively retained and all the contaminants (proteins, RNA and low molecular weight molecules) eluted by adding 30 mL of washing solution (1M NaCl, 50 mM MOPS pH7.0, 15% isopropanol (V/V)) twice. To elute the molecules of interest, the positive charge of the columns was neutralized with a change of pH to slightly alkaline conditions and then eluted by an increment of the salt concentration using the 15 mL of the elution solution (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol (V/V)). The nucleic acids were then precipitated using 0.7 volumes of cold isopropanol and then incubated on ice for 30 minutes, performing after a centrifugation at 16 000g during 30 minutes at 4 ºC. After discarding the supernatant, the pellet was resuspended using 1 mL of 10 mM Tris-EDTA (pH 8.0) buffer. The final samples were quantified through the nanophotometer (Implen GmbH, Germany) and stored at -80 ºC, for posterior use on transfection assays. Band densitometry analysis was performed using ImageJ software (NIH, United States of America).

3.2.2 Modified alkaline lysis

In order to obtain lysate samples of mcDNA to be applied in the size exclusion chromatography, a modified alkaline lysis was performed. For such, the bacteria pellet recovered after fermentation equivalent to a volume of 250 mL was resuspended in 20 mL of solution A (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0), being the alkaline lysis performed by the addition of 20 mL of solution B (200 mM NaOH, 1% SDS (w/V)) and incubated during 5 minutes at room temperature. To neutralize this reaction, 20 mL of solution C (3 M potassium acetate, pH 5.0) was added and incubated for a period of 20 minutes on ice. The elimination of cell debris was achieved through two centrifugation steps at 20 000g, during 30 minutes at 4 ºC, in the Allegra™ 25R centrifuge (Beckman Coulter, United States of America). The nucleic acids on the supernatant were precipitated by adding 0.7 volumes of cold isopropanol and incubated on ice for 30 minutes. Up next, a centrifugation at 16 000g, during 30 minutes at 4 ºC was performed. After disposing of the supernatant, the resulting pellet was resuspended in 2 mL of 10 mM Tris-EDTA buffer (pH 7.0). To precipitate proteins and RNA, ammonium sulphate was added for a final concentration of 2.5 and incubated 15 minutes on ice. These impurities were removed through centrifugation at 16 000g, during 15 minutes at 4 ºC, recovering the supernatant. The final samples were quantified through a nanophotometer (Implen GmbH, Germany) and stored at -80 ºC, for further used in size exclusion chromatography.

3.3 Purification

3.3.1 Size exclusion chromatography

The size exclusion chromatography was another strategy explored with the aim of promoting the separation of the parental plasmid from the minicircular DNA and RNA, accordingly to its molecular size differences, starting with samples from modified alkaline lysis. For such, two columns of Sephacryl S-100 SF (GE Healthcare Biosciences, Sweden) of 60 and 90 cm, with volumes of 106 and 180 mL, respectively, were used and equilibrated with 10 mM Tris, 10 mM EDTA, 150 mM NaCl (pH 7.0), performing the assay with a flow of 0.3 mL/min. The volume of injected lysate sample was of 2 mL, both for the 60 and 90 cm columns, and fractions of 3 mL were collected throughout the assay. The chromatographic assay was performed at room temperature and the absorbance was monitored at 260 nm. All the collected fractions were desalted and concentrated to a final volume of 200 µL using Vivaspin® concentrators for posterior results analysis through agarose gel electrophoresis. Lastly, the fractions presenting mainly mcDNA were selected and quantified using NanoPhotometer (IMPLEN, DE).

3.3.1.1 Agarose gel electrophoresis

The chromatographic fractions were analysed through agarose gel electrophoresis 1% (w/V) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The gel was stained with 0.016 µL/mL of GreenSafe Premium reagent (NZYTech, Genes & Enzymes, Portugal). The electrophoresis run was performed during 30 minutes at 110V and the gel visualized under UV light in a transilluminator UVITEC Fire-Reader (UVITEC, United Kingdom).

3.4 *in vitro* assays

3.4.1 Cell lines and culture conditions

The CaSki cells (metastatic cells from the cervical epidermoid carcinoma, HPV-16) were cultured in RPMI medium (Roswell Park Memorial Institute) and the human fibroblasts were cultured in DMEM/F-12 medium (Dulbecco's Modified Eagle's Medium/Ham's F-12 Nutrient Mixture). These culture mediums (Sigma-Aldrich, United States of America) were supplemented with 10% (V/V) fetal bovine serum (FBS) and with 1% (V/V) with a mixture of antibiotics composed of penicillin (100 µg/mL) and streptomycin (100 µg/mL). The cellular growth was promoted at 37 ºC, in a moisture atmosphere containing 5% of CO2 and 95% of air.

3.4.2 Transfection

As the main goal of the *in vitro* studies was to evaluate the therapeutic effect of the encoding gene present in different vectors (mcDNA-p53, mcDNA-primiR-375 and mcDNA-primiR-375+p53). For such, a transfection reagent Torpedo^{DNA} (ibidi GmbH, Germany) was used according to the manufacturer's instructions. The cells were maintained in culture until an 50-60% confluence was achieved, being seeded in plates with a density of 3 x 10^4 and 6.2 x 10^4 cells/cm^2 for the human fibroblasts and CaSki cells, respectively, in complete medium. After 24 hours of culture, the complete medium was replaced with incomplete medium without antibiotic. In the following day, the transfection reagent was incubated with the vectors during 20 minutes at room temperature, in order to assure the formation of complexes between cationic lipids and the DNA. The DNA (µg) : Torpedo^{DNA} (µL) ratio used was of 1:3, according to the manufacturer protocol. The reactional mixtures were added to the respective wells and the transfected cells were once again incubated with the same culture conditions. After 6 hours of the beginning of the transfection, the incomplete medium without antibiotic was replaced by complete medium.

3.4.3 Cellular cytotoxicity evaluation assay

The cytotoxicity of the vectors was evaluated through the resazurin assay. CaSKi and human fibroblast cells were seeded with a density of 2.06 x 10^4 and 10^4 cells/well, respectively and the transfections were performed during 24, 36, 48 and 72 hours. Two hours prior to the end of each transfection period, the culture medium was discarded and 100 µL of fresh complete medium and 20 µL of resazurin 0.1% (w/V) were added to each well and incubated for four hours in the dark, using the same culture conditions. After this period of incubation, the reactional mixture was transferred into an opaque plate. The fluorescence was measured in a spectrofluorometer (SpectraMAX® Gemini™ EM, Molecular Devices, United States of America), defining two different wavelengths, $\lambda_{excitation}$ = 544 nm and $\lambda_{emission}$ = 590 nm, to analyse the resofurin fluorescence produced. Non-transfected cells were used as negative control and cells treated with 70% ethanol were used as a positive control. The assays were performed with a n = 3 and the statistical analysis was performed with the two-way ANOVA, using GraphPad Prism software (version 6.01).

3.4.4 Proliferation assay

Cellular proliferation was evaluated over time, through the number of viable cells counting. CaSki cells were seeded with a density of 1.17 x 10^4 cells/well in plates of 24 wells. The transfection with the vectors under study was performed during 24, 36, 48 and 72 hours. To stop the transfection, the culture medium was discarded, and trypsin was added to each well. Posteriorly, the cells were recovered through centrifugation and the number of adherent viable cells was determined using the exclusion method through trypan blue. Non transfected

cells and cells transfected with the vector that does not contain any sequence encoding genes were used as controls. The assays were performed to a n = 3 and the statistical analysis was performed with two–way ANOVA, using GraphPad Prism software (version 6.01).

3.4.5 Protein extraction

The CaSki cells were seeded with a density of 5 x 10^5 cells/well on a plate of 6 wells (VWR International, United States of America). After 48 hours of transfection with the vectors under study, the non-transfected (control) and transfected cells were recovered and submitted to protein extraction. Firstly, the culture medium was removed, and each well was washed with PBS. Up next, the cells were recovered using a cell scraper and centrifugated at 11 500 rpm, during 10 minutes at 4 ºC on a Hettich Mikro 200R centrifuge (Andreas Hettich GmbH, Germany). The supernatant was disposed, and lysis buffer was added (Roche, United States of America). This lysis buffer is composed with 25 mM Tris (pH 7.4), 2.5 mM EDTA, 2% Triton X-100, 2.5 mM EGTA, 1 mM PMSF and 10 µL/mL of protease inhibitor without EDTA cocktail. Finally, a 10 minutes incubation on ice was performed. To eliminate cellular debris, an additional centrifugation at 11 500 rpm, for 10 minutes at 4 ºC was performed.

3.4.5.1 Protein quantification

After the extraction, the BCA kit was used to determine protein concentration. Beginning with a BSA standard of 2 mg/mL from the commercial kit, a series of dilutions ranging from 0 to 1200 µg/mL was prepared according to table 1. Next, 80 µL of working reagent was added to each well.

Table 4. Volumes needed to prepare BSA standards.

BSA standard (µg)	BSA (µL)	Extraction buffer (µL)	Mili-Q water (µL)
0	0	1	19
2	1	1	18
4	2	1	17
6	3	1	16
8	4	1	15
10	5	1	14
12	6	1	13
Total	20 µL		

For the protein quantification in the sample, in a microplate triplicates were done with 1 µL of protein extracted sample and 19 µL of Mili-Q water. For the blank, 19 µL of water and 1 µL of the extraction reagent was added to each well. For both, 80 µL of working reagent was added to each well. The microplate was protected from the light and incubated at 37 ºC for 30 minutes and the absorbance was measured at 562 nm.

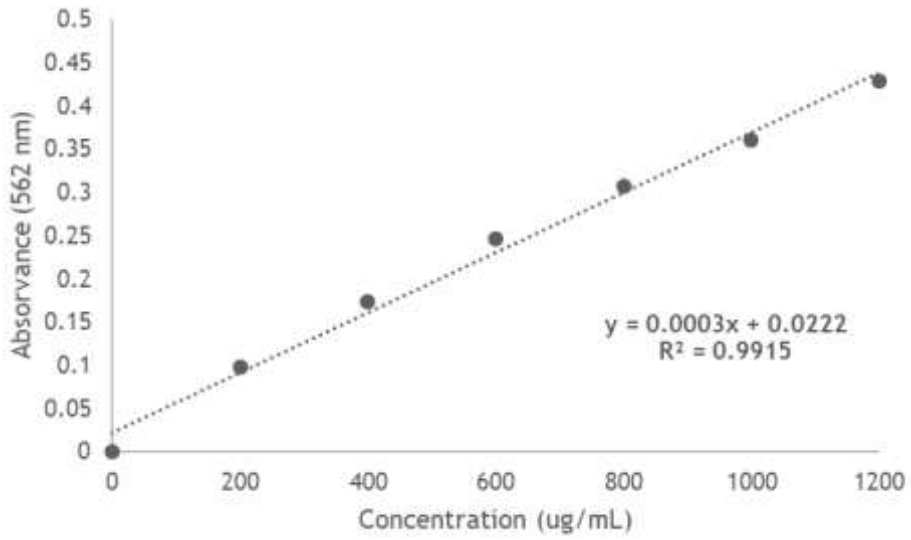

Figure 9. Standard curve with BSA standard protein 0 – 1200 µg/mL.

3.5 Western-Blot

After protein extraction and quantification, a polyacrylamide gel electrophoresis was performed in denaturing conditions, named SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). This technique goal is to separate the proteins based on its molecular weight. In this way, the samples were prepared with a total protein concentration of 30 µg, adding loading buffer in a ratio of 4:1. The samples were denaturated for 10 minutes at 100 ºC, injected in a 4.7% stacking acrylamide gel and then separated according to its molecular weight in a 12.5% acrylamide resolving gel. The molecular weight marker used was the NZYColour Protein Marker II (NZYTech – Genes and Enzymes, Portugal). The electrophoresis run was performed at 95V until the samples reached the resolving gel and then the voltage was increased to 115V during 90 minutes at room temperature. The electrotransfer consists of a protein transference from de polyacrylamide gel to a PVDF membrane, previously activated with methanol, through the application of an electrical current of 750 mA, during 90 minutes at 4 ºC. Afterwards, the membrane was washed 3 times with TBS buffer containing 0.1% of Tween 20 (TBS-T 0.1%), for 5 minutes and then blocked with a 5% (w/V) BSA solution during 1 hour at room temperature under constant agitation, to prevent unspecific interactions between the membrane and the antibody used to detect the target protein. The membrane was washed once again 3 times for 5 minutes in order to incubate with the monoclonal antibodies anti-p53, anti-E6, anti-E7 and anti-ß-actin (Santa Cruz Biotechnology, Germany) overnight at 4 ºC, under constant agitation. After each specific incubation, the membrane was washed again, using the same procedure as before so that the polyclonal secondary antibody IgG anti-rabbit or anti-mouse (Santa Cruz Biotechnology, Germany) for 1 hour at room temperature with constant agitation. After washing the membrane, incubation with 200 µL of ECL was performed (Advansta Inc., United States of

America) for 5 minutes. The membrane was visualized on the BioRad ChemiDoc system (BioRad, United States of America).

Chapter IV – Results and Discussion

4.1 Parental plasmid growth curve

The aim of this dissertation is to study the potential therapeutic effect of three vectors encoding for different genes, namely p53, primiR-375 and primiR-375+p53. These different vectors present different sizes, 5 Kbp, 4.5 Kbp and 5.7Kbp, respectively. In addition, the same vector without gene, denominated mcDNA-empty, was also used as a control, presenting a size of 3.8 Kbp.

To achieve the main goal of this dissertation, ZYCY10P3S2T *E. coli* strain harboring the CMV-MCS-EF1-GFP-SV40 PolyA vector (PP-empty) and the CMV-MCS-EF1-GFP-SV40 PolyA vector modified with the p53, primiR-375 and both genes (PP-p53, PP-primiR-375 and PP-primiR-375+p53) was constructed, followed by the fermentation of these bacteria. The parental plasmids final size was of 7 Kbp, 8.2 Kbp, 7.7 Kbp and 9.5 Kbp for the PP-empty, PP-p53, PP-primiR-375 and PP-primiR-375+p53, respectively.

Based on the different sizes presented by the vectors, measurements of the fermentation optical density at 600 nm (this is, in the visible range, where we can detect the bacteria) were made during the fermentation, in order to understand if the different vector sizes would be responsible for presenting or not different kinetics growth profiles.

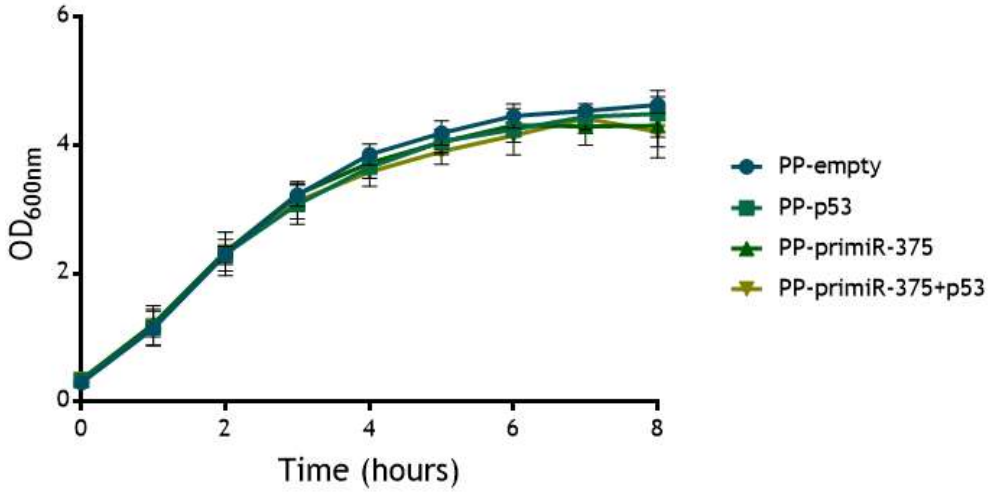

Figure 10. Graphical representation of the growth curve built by the measurement of the optical density of the fermentation medium of the different vectors in study every hour at 600 nm. The measurements were made for a n = 3 and the statistical analysis was performed using one-way ANOVA with GraphPad software.

From the analysis of the growth curves (Figure 10), especially by its overlapping and in spite of their different sizes, of the *E. coli* ZYCY10P3S2T transformed with the PP vectors in the study demonstrated to have a very similar kinetic profile, suggesting that the different sizes

presented by the vectors were not preponderant for their growth profile. Cheah and co-workers studied the effect of plasmid size on *E. coli* growth[77]. Their findings revealed that regardless of the plasmid size, the growth profile was very similar.

4.2 Extraction

After the different PPs fermentation, the bacteria were submitted to L-Arabinose inductor, aiming to obtain the recombination of PPs into the respective mcDNAs. The mcDNA vectors were produced with the intention of being applied in gene therapy, so they must be purified in order to eliminate the presence of contaminants, such as genomic DNA (gDNA) or RNA, not allowed in pharmaceutical formulations by the regulatory agencies (EMA and FDA). These agencies establish the following impurity criteria: gDNA/pDNA under 0.01 µg/µg, protein and RNA content undetectable and endotoxins/pDNA lower than 40 EU/mg[78].

4.2.1 Kit Qiagen® - manufacturer protocol

The first approach to purify the mcDNA samples was to apply a commercial extraction and purification kit, Plasmid Purification DNA Kit by QIAGEN®, usually used to purify plasmid DNA (pDNA). This strategy was applied to the primiR-375 and primiR-375+p53 lysate samples, the smallest and the biggest mcDNA vectors in study. During the extraction and purification protocol, samples of each stage were collected and analyzed by agarose gel electrophoresis (Figure 11).

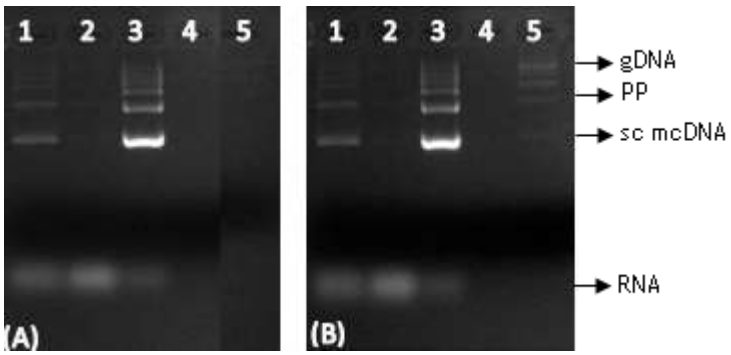

Figure 11. Agarose gel electrophoresis of the extraction and purification according to the manufacturer protocol of the mcDNA (A) primiR-375 and (B) primiR-375+p53 vectors. 1 - lysate; 2 - supernatant; 3 - washing step I; 4 - washing step II; 5 - final sample.

The concentration of the final sample was 12 and 4.5 µg/mL for the mcDNA-primiR-375 and primiR-375+p53 vectors, respectively. These low concentrations can be justified through the agarose gel electrophoresis results analysis (Figure 11), for both vectors, where it is observed in lane 3, corresponding to the washing step I, that the majority of mcDNA was eluted. The elution of the mcDNA in the washing step may be caused by the ionic strength conditions of the washing buffer (1M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (V/V)) not being suitable to maintain the mcDNA linked to the positively charged matrix, washing only the sample

impurities, leading to an almost total elution of the nucleic acids present in the lysate sample.

As a low yield of interest mcDNA molecules was obtained using the manufacturer protocol, and still based on the solutions and procedure of this kit, some optimizations were performed in order to understand if better mcDNA recovery yields would be obtained. The salt concentration in the washing and elution solutions was decreased to 500 mM and augmented to 1.75 M, respectively.

4.2.2 Kit Qiagen® - optimized protocol

The optimizations made were the decreasing of salt concentration in the washing solution (500 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol (V/V)), in order to promote mcDNA interaction with the anion exchange chromatographic column. On the other hand, in the elution solution, the salt concentration was raised so that the elution of the interest molecules is promoted (1.75 M NaCl, 50 mM Tris-HCl pH 8.5, 15 % isopropanol (V/V)). Throughout the purification procedure, samples of each step were collected and analyzed by agarose gel electrophoresis (Figure 12).

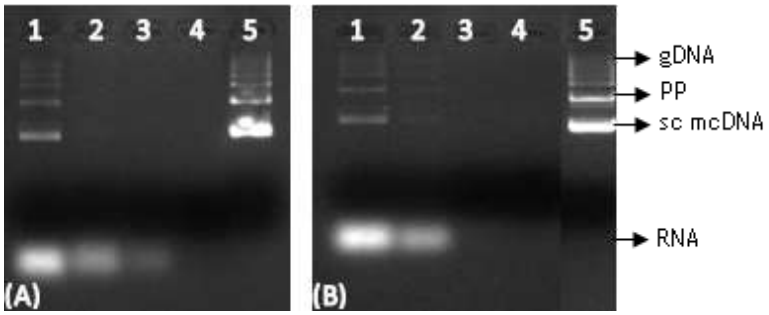

Figure 12. Agarose gel electrophoresis of the extraction and purification optimized protocol of the mcDNA (A) primiR-375 and (B) primiR-375+p53 vectors. 1 - lysate; 2 - supernatant; 3 - washing step I; 4 - washing step II; 5 - final sample.

From the analysis of the agarose gel electrophoresis (Figure 12), it is possible to observe that contrarily to the one obtained following the manufacturer protocol (Figure 11), in the washing step I (lane 3) none of the interest sample is lost. In addition, it is possible to see that RNA is the only molecule eluting in the washing step I (lane 3), as it is expected. Moreover, by the analysis of Figure 12, it is also noticeable that during the entire procedure none of the mcDNA was lost, eluting only after the use of the elution solution as desired, proving that the alterations in the composition of the solutions were adequate and efficient to retain DNA molecules in the anionic column.

The final concentration of the samples obtained with the optimized protocol was of 58 and 72.5 µg/mL for the mcDNA-primiR-375 and primiR-375+p53 vectors (Table 5), respectively, significantly higher than the concentrations obtained by using the manufacturer's protocol.

Table 5. Final mcDNA-primiR-375 and mcDNA-primiR-375+p53 concentration following the optimized protocol and respective band intensities for the mcDNA and impurities.

	Final concentration (µg/mL)	mcDNA band intensity (%)	Impurities band intensity (%)	mcDNA mass (µg)
mcDNA-primiR-375	58	49	51	28.42
mcDNA-primiR-375+p53	72.5	41	59	29.73

Even though the concentration of the final samples has improved, there is still a drawback. This purification technique does not allow the separation of the mcDNA from the PP that was not intramolecularly converted in the induction stage and also some gDNA contamination can be observed. Through band intensity analysis, using ImageJ software (NIH, United States of America) to evaluate the amount of contamination with gDNA and PP in the final sample after the purification technique, the percentage of gDNA and PP contamination in the final sample was of 51% and 59% for the mcDNA-primiR-375 and mcDNA-primiR-375+p53, respectively (Table 5), that calculating the mcDNA mass, this technique allowed a recovery of 28.42 and 29.73 µg, respectively. So, in order to overcome this contamination degree, a different purification technique was used.

In the next approach, firstly an extraction of the mcDNA vectors using a modified alkaline lysis protocol was performed, allowing not only the nucleic acids extraction but also a pre-clarification (once, these technique includes steps where the proteins and part of the RNA will be precipitated and eliminated) of the final sample to be purified using size exclusion chromatography.

4.2.3 Modified extraction

In the modified extraction strategy, there is no use of solutions containing RNase in their composition, contrarily to the commercial kit procedure. Taking into consideration that the regulatory agencies do not recommend the application of enzymes of animal origin[39] during the extraction of biopharmaceuticals for therapeutic applications, this methodology already presents an advantage over the previous. As such, the final sample obtained is not exempt from RNA. However, to simplify the lysate sample applied to the size exclusion chromatography, the sample was primarily concentrated using isopropanol (0.7 V) for the nucleic acids precipitation and then the sample was clarified due to the elimination of proteins and high molecular weight RNA by precipitation with high ammonium sulphate concentration (2.5 M).

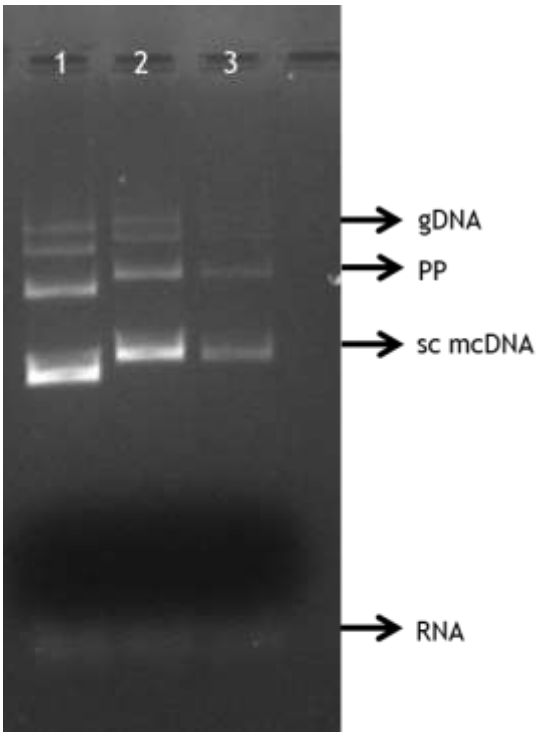

Figure 13. Agarose gel electrophoresis of the samples obtained after the modified extraction. 1– mcDNA-primiR-375; 2 – mcDNA-p53; 3 – mcDNA-primiR-375+p53.

In the Figure 13 is represented the agarose gel electrophoresis of the lysate samples obtained for the mDNA-primiR-375, mcDNA-p53 and mcDNA-primiR-375+p53 using this methodology. This result shows that even though a significant reduction of the RNA content and the mcDNA band is more intense in comparison to the PP and gDNA, the final lysate sample is still very contaminated by these molecules, reinforcing the need to perform a purification procedure.

4.3 Size exclusion chromatography

The sample obtained in the modified extraction step was injected in a sepharose column, Sephacryl S-1000 SF, of 60 and 90 cm, depending on the size of the mcDNA vector, in order to perform size exclusion chromatography. This matrix was chosen due to its wide fractionation range (between 5 x 10^5 up to 10^8 molecular weight), which may allow the separation of macromolecules (gDNA, PP and mcDNA), as previously described by Almeida and co-workers[78].

This purification technique is based on the separation of the sample molecules according to its molecular weight differences. Thus, the separation of the different molecules occurs in descending order of molecular weight, eluting first the molecules of higher molecular weight, in this case the gDNA, then the PP, followed by the mcDNA and finally the RNA. This difference in the elution is due to the smaller molecules take a larger route once they can enter the sepharose spheres pores, getting delayed in comparison to the larger ones, eluting in last[69].

To evaluate the separation of the molecules, throughout the entire purification procedure, fractions of 3 mL were collected, desalted using a 10 mM Tris-EDTA pH 7.0 buffer and

concentrated to a final volume of 200 µL prior being injected into the agarose gel for electrophoresis analysis allowing the selection of those presenting mainly sc mcDNA. The selected samples were then quantified using the NanoPhotometer (Imple GmbH, Germany) and stored at -80 ºC.

Almeida and co-workers[78] tested several flows and volumes of fractions collected throughout size exclusion chromatography using a 121 mL Sephacryl S-1000 SF column. Their results showed that using a 0.3 mL/min flow allowed the recovery of mcDNA fractions less contaminated in comparison to the other flows studied, being the reason why this flow was chosen to perform SEC assays. All chromatographic experimentations were performed using AKTA Püre system (GE Healthcare, Buckinghamshire, UK), that consists in a compact separation unit and personal computer with UNICORN™ 6.3 software.

4.3.1 mcDNA-primiR-375

The size exclusion chromatography assay was performed, using a 106 mL Sepharose column previously equilibrated using 10 mM Tris-EDTA, 150 mM NaCl pH 7.0 buffer. A sample volume of 2 mL was injected directly onto de column and was used an isocratic gradient with the equilibrium buffer 10 mM Tris-EDTA, 150 mM NaCl pH 7.0, with a flow of 0.3 mL/min at room temperature until all the sample molecules are eluted. Throughout the entire assay, the absorbance was continuously monitored at 260 nm.

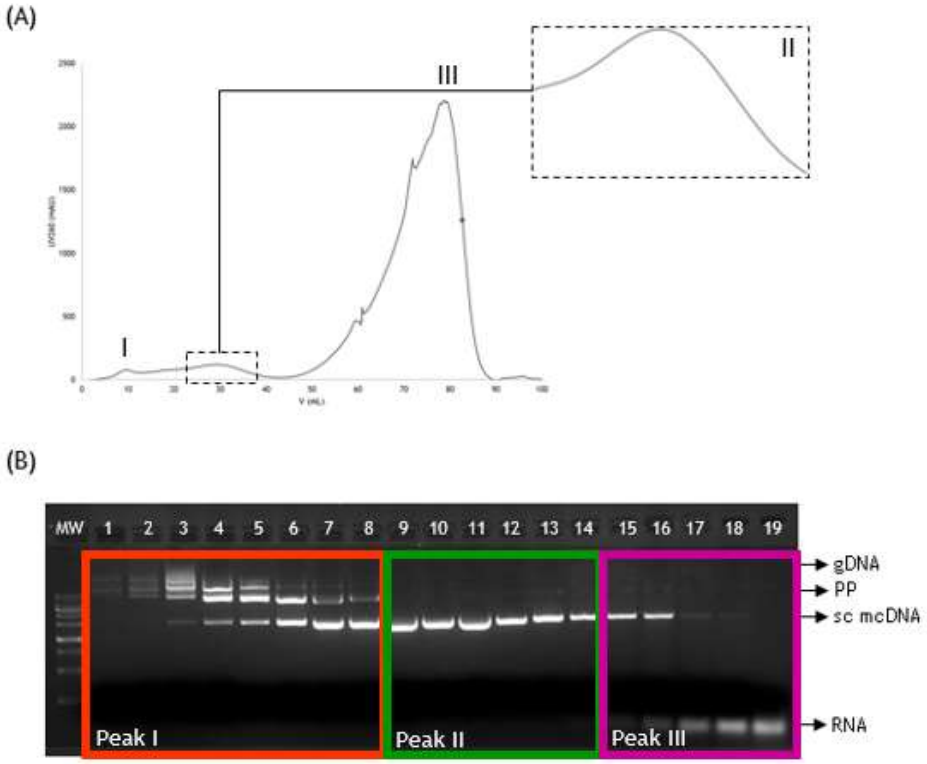

Figure 14. Size exclusion chromatography (A) and agarose gel electrophoresis (B) of the mcDNA-primiR-375 vector. MW – molecular weight.

Analyzing the chromatogram of the mcDNA-primiR-375 (4.5 Kbp) size exclusion chromatography assay, Figure 14 (A), it is possible to observe the presence of three peaks, that comparing to the agarose gel electrophoresis, Figure 14 (B), corresponds to the elution of the gDNA and PP in the peak I, between 8 and 13 mL, followed by the elution of the sc mcDNA in the peak II, between 22 and 38 mL and finally the elution of the RNA in the peak III that starts at 50 mL of assay.

Furthermore, through the agarose gel electrophoresis analysis, Figure 14 (B), it is possible to observe the presence of mcDNA in the fractions 3 – 16. However, the fractions 3 – 6 are very contaminated with gDNA and PP, likewise to the fractions 15 and 16 that have RNA contamination. Once the fractions 7 to 14 are the ones composed mainly by sc mcDNA-primiR-375, were selected for posterior *in vitro* transfection assays. These selected fractions were then quantified using Implen Nanophotometer and the mcDNA mass was calculated (Table 6).

Table 6. Representation of the concentration of fractions collected and desalted during the mcDNA-primiR-375 size-exclusion chromatographic assay, using a 106 mL column and respective mcDNA mass of the selected fractions.

Fraction	Concentration (µg/mL)	mcDNA mass (µg)	Fraction	Concentration (µg/mL)	mcDNA mass (µg)
1	5.5		11	120	24
2	6.5		12	45	9
3	25.5		13	22.5	4.5
4	40.5		14	16	3.2
5	45.5		15	26.5	
6	40		16	42.5	
7	70.5	14.1	17	46	
8	57.7	11.54	18	55	
9	95.5	19.1	19	141	
10	77	15.4	Total		100.84

Based on the results present in Table 6, after the purification of the mcDNA-primiR-375 sample, a mass of 100.84 µg of sc mcDNA was obtained with a high purity degree. In comparison to the final nucleic acids mass recovered using commercial kit optimized protocol, 28.42 µg, it is possible to conclude that comparing both purification strategies exploited for the mcDNA purification, besides the mcDNA recovered using SEC be almost the double than the one recovered using the optimized commercial kit protocol, this strategy also allowed the recovery of only mcDNA, not possible using the commercial kit once the final sample in this case is contaminated with gDNA and PP. These results showed that the use of a

106 mL sepharose column was efficient to obtain samples mainly composed of sc mcDNA primiR-375.

Taking into consideration the results obtained using SEC for mcDNA-primiR-375 purification (Figure 14) and comparing to the ones that Almeida and co-workers[78] obtained using the same flow rate and sample volume injected, is possible to conclude that in both cases, the mcDNA fractions collected and selected for posterior *in vitro* transfection assays, were obtained less contaminated with gDNA and PP, therefore being considered a suitable strategy for the mcDNA purification.

4.3.2 mcDNA-p53

For the size exclusion chromatography assay performed to purify the mcDNA-p53, similarly to the mcDNA-primR-375, a 106 mL Sepharose column previously equilibrated with 10 mM Tris-EDTA, 150 mM NaCl pH 7.0 buffer, was used. A sample volume of 2 mL was injected directly onto de column and was used an isocratic gradient with the equilibrium buffer 10 mM Tris-EDTA, 150 mM NaCl pH 7.0, with a flow of 0.3 mL/min at room temperature until all the sample molecules are eluted, monitoring during the whole assay, the absorbance was continuously monitored at 260 nm.

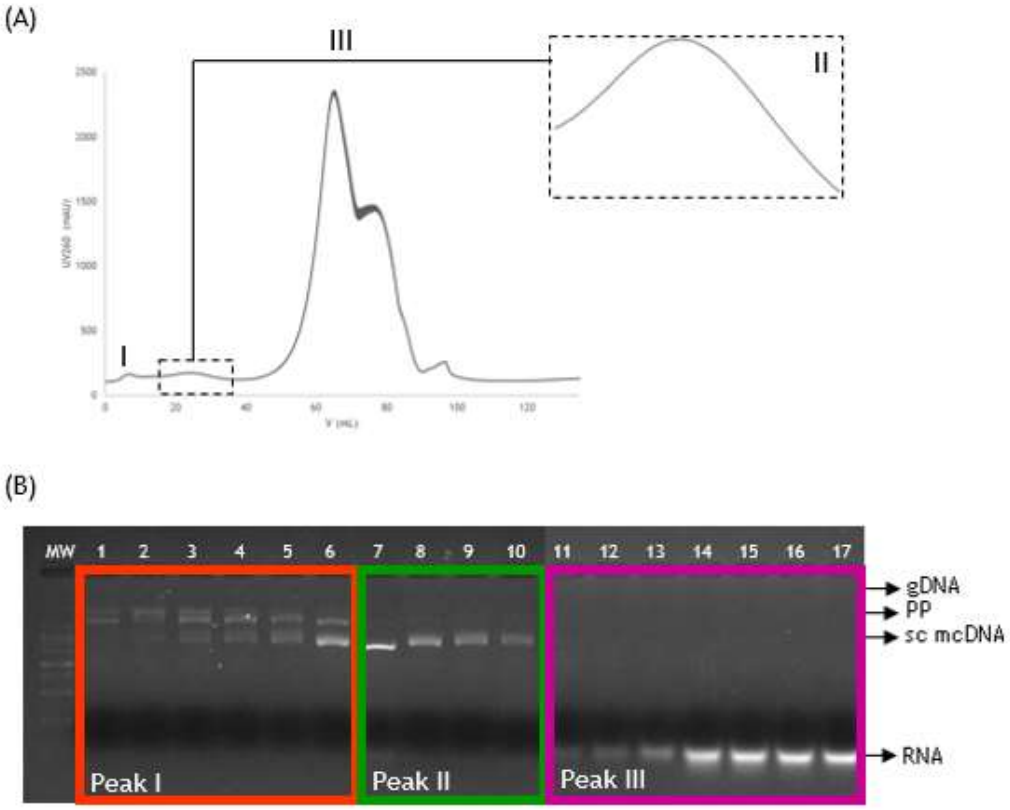

Figure 15. Size exclusion chromatography (A) and agarose gel electrophoresis (B) of the mcDNA-p53 vector. MW – molecular weight.

Evaluating the chromatogram of the mcDNA-p53 (5 Kbp) size exclusion chromatography assay, Figure 15 (A), it is possible to observe the presence of three peaks, that comparing to the agarose gel electrophoresis, Figure 15 (B), corresponds to the elution of the gDNA and PP in the peak I, between 6 and 13 mL, followed by the elution of the sc mcDNA in the peak II, between 20 and 36 mL and finally the elution of the RNA in the peak III that starts at 48 mL of assay.

Moreover, through the agarose gel electrophoresis analysis, Figure 15 (B), it is possible to observe the presence of mcDNA in the fractions 3 – 10. However, the fraction 3 – 6 are very contaminated with gDNA and PP. The fractions 7 to 10 present only sc mcDNA-p53, being those selected for posterior i*n vitro* transfection assays. These selected fractions were quantified using Implen Nanophotometer and the mcDNA mass was calculated (Table 7).

Comparing the mcDNA yield obtained for the mcDNA-primiR-375 (Table 5), the use of SEC sepharose column for purification of mcDNA-p53 was not as well succeed (Table 6), once the final mcDNA-p53 selected fractions for posterior *in vitro* assays have only 36 µg.

Table 7. Representation of the concentration of fractions collected and desalted during the mcDNA-p53 size-exclusion chromatographic assay, using a 106 mL column and respective mcDNA mass of the selected fractions.

Fraction	Concentration (µg/mL)	mcDNA mass (µg)	Fraction	Concentration (µg/mL)	mcDNA mass (µg)
1	14		10	19	3.8
2	18.5		11	64.5	
3	19		12	105	
4	17.5		13	152	
5	21.5		14	295	
6	29	5.8	15	381	
7	85	17	16	504	
8	26	5.2	17	642	
9	21	4.2	Total		36

4.3.3 mcDNA-primiR-375+p53

The size exclusion chromatography assay was also performed to isolate the mcDNA-primiR-375+p53, using a 106 mL Sepharose column previously equilibrated using 10 mM Tris-EDTA, 150 mM NaCl pH 7.0 buffer. A sample volume of 2 mL was injected directly onto de column and was used an isocratic gradient with the equilibrium buffer 10 mM Tris-EDTA, 150 mM NaCl pH 7.0, with a flow of 0.3 mL/min at room temperature until all the sample molecules are eluted. Throughout the entire assay, the absorbance was continuously monitored at 260 nm.

Analyzing the chromatogram of the mcDNA-primiR-375 (5.7 Kbp) size exclusion chromatography assay, Figure 16 (A), it is possible to observe the presence of three peaks, that comparing to the agarose gel electrophoresis, Figure 16 (B), corresponds to the elution of the gDNA and PP in the peak I, between 6 and 10 mL, followed by the elution of the sc mcDNA in the peak II, between 16 and 34 mL and finally the elution of the RNA in the peak III that starts at 45 mL of assay.

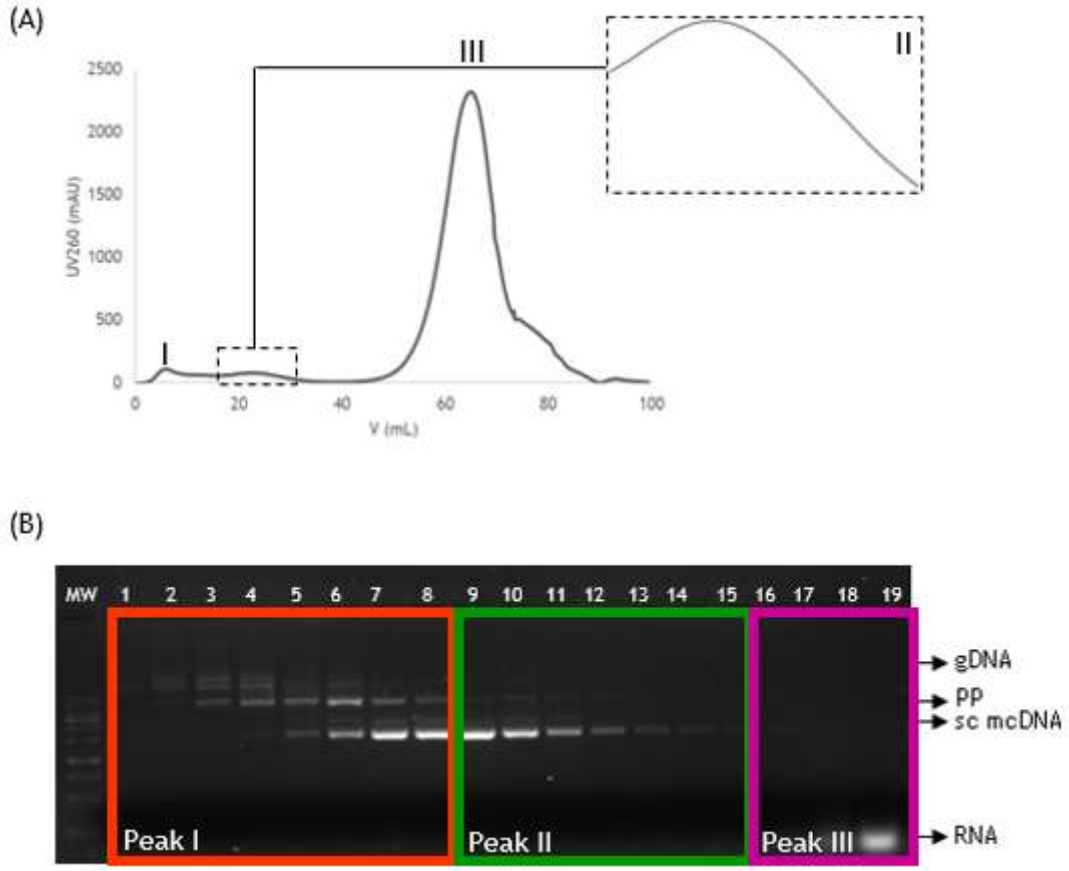

Figure 16. Size exclusion chromatography chromatogram using a 106 mL column (A) and agarose gel electrophoresis (B) of the mcDNA-primiR-375+p53 vector. MW – molecular weight.

Additionally, through the agarose gel electrophoresis analysis, Figure 16 (B), it is possible to observe the presence of mcDNA in the fractions 3 – 14. Nevertheless, the fraction 3 – 6 are very contaminated with gDNA and PP, therefore fractions 9 to 14 that are composed mainly by sc mcDNA-primiR-375+p53, were the ones selected for posterior i*n vitro* transfection assays. These selected fractions were quantified using Implen Nanophotometer and the mcDNA mass was calculated (Table 8).

Grounded on the results present in Table 8, after the purification of the mcDNA-primiR-375+p53 sample, a mass of 36.6 µg of sc mcDNA was obtained with a high purity degree. Comparing the mcDNA-primiR-375+p53 mass obtained with both purification strategies exploited, the yield obtained with the optimized protocol of the commercial kit was superior,

29.73 µg. Nonetheless, SEC showed to allow the recovery of only mcDNA, contrarily to the commercial kit which final sample is contaminated with PP.

Table 8. Representation of the concentration of fractions collected and desalted during the mcDNA-primiR-375+p53 size-exclusion chromatographic assay, using a 106 mL column and respective mcDNA mass of the selected fractions.

Fraction	Concentration (µg/mL)	mcDNA mass (µg)	Fraction	Concentration (µg/mL)	mcDNA mass (µg)
1	13		11	29	5.8
2	16.5		12	17.5	3.5
3	24.5		13	15.5	3.1
4	34.5		14	14.5	2.9
5	38		15	27.5	
6	44.5		16	42	
7	75		17	52	
8	61.5		18	135	
9	63	12.6	19	318	
10	43.5	8.7	Total		36.6

The loss of mcDNA-p53 and mcDNA-primiR-375+p53 (5 and 5.7 Kbp, respectively) may be due to its higher molecular weight in comparison to the mcDNA-prmiR-375 (4.5 Kbp), leading to an earlier elution time (the mcDNA elution period starts at 72, 66 and 60 minutes, for the mcDNA-primiR-375, mcDNA-p53 and mcDNA-primiR-375+p53, respectively), causing to some of the mcDNA eluting along with the PP, so there is the need to discard these fractions to avoid a final mcDNA fraction contaminated.

In order to overcome the low mcDNA mass recovery using a 60 cm sepharose column, and taking into consideration the parameters in SEC that can be manipulated so that a more efficient separation can be achieved, in particular, column size, flow and volume of sample injected[69], some adjustments were made.

Almeida and co-workers[78], exploited several strategies to try to improve mcDNA recovery with a high purity. They explored the use of a 60 cm column with different flow rates, 0.2 and 0.3 mL/min and the use of a 90 cm column with a 0.3 mL/min flow rate. Their findings showed a better mcDNA separation of higher molecular weight from the contaminants using a 90 cm column with a flow rate of 0.3mL/min. As such, a 90 cm Sepharose column was then used to try to understand if a better mcDNA yield was obtained applying the same strategy (Figure 17).

Once again, the size exclusion chromatography assay was performed, using a 180 mL sepharose column previously equilibrated using 10 mM Tris-EDTA, 150 mM NaCl pH 7.0 buffer and a sample volume of 2 mL was injected directly onto de column and was used an isocratic gradient with the equilibrium buffer 10 mM Tris-EDTA, 150 mM NaCl pH 7.0, with a flow of 0.3 mL/min at room temperature until all the sample molecules are eluted. Throughout the entire assay, the absorbance was continuously monitored at 260 nm.

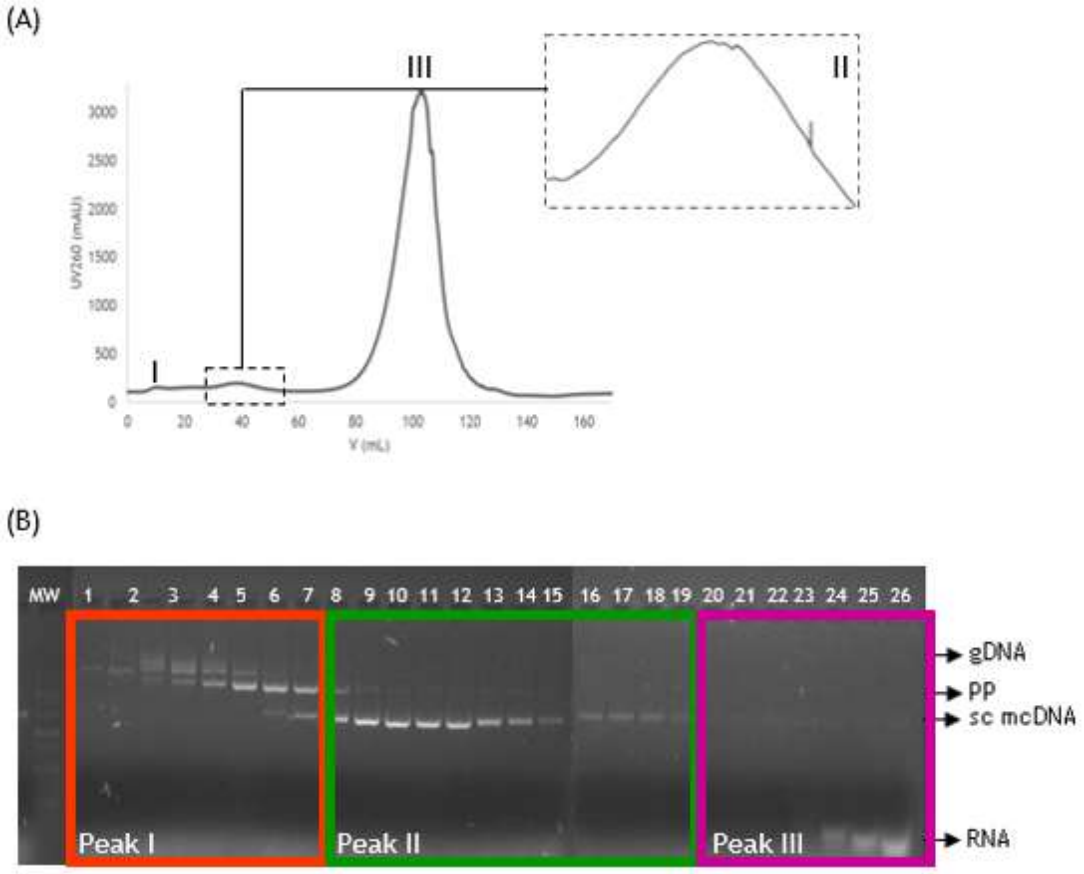

Figure 17. Size exclusion chromatography chromatogram using a 180 mL column (A) and agarose gel electrophoresis (B) of the mcDNA-primiR-375+p53 vector. MW - molecular weight.

Analyzing the chromatogram of the mcDNA-primiR-375+p53 (5.7 Kbp) size exclusion chromatography assay using the 90 cm sepharose column, Figure 17 (A), it is possible to observe the presence of three peaks, that comparing to the agarose gel electrophoresis, Figure 17 (B), corresponds to the elution of the gDNA and PP in the peak I, between 8 and 18 mL, followed by the elution of the sc mcDNA in the peak II, between 26 and 58 mL and finally the elution of the RNA in the peak III that starts at 70 mL of assay.

Additionally, through the agarose gel electrophoresis analysis, Figure 17 (B), it is possible to observe the presence of mcDNA in the fractions 3 – 19. Though, the fraction 3 – 7 are very contaminated with gDNA and PP. The fractions 8 to 19 are composed mainly by sc mcDNA-primiR-375+p53, being those selected for posterior i*n vitro* transfection assays. These

selected fractions were quantified using Implen Nanophotometer and the mcDNA mass was calculated (Table 9).

Table 9. Representation of the concentration of fractions collected and desalted during the mcDNA-primiR-375+p53 size-exclusion chromatographic assay, using a 180 mL column and respective mcDNA mass of the selected fractions.

Fraction	Concentration (µg/mL)	mcDNA mass (µg)	Fraction	Concentration (µg/mL)	mcDNA mass (µg)
1	3.5		16	49.5	9,9
2	5.5		17	34	6,8
3	5.5		18	28.5	5,7
4	6		19	14	2.8
5	10		20	11.5	
6	20		21	11.5	
7	24		22	9.5	
8	27.5	5.5	23	9	
9	27.5	5.5	24	8.5	
10	36	7.2	25	6	
11	42	8.4	26	11	
12	42	8.4	27	106	
13	50.5	10.1	28	508	
14	57	11.4	29	838	
15	64.5	12.9	30	1049	
			Total		94.6

Based on the results present in Table 9, after the purification of the mcDNA-primiR-375+p53 sample using a 90 cm column, a mass of 94.6 µg of sc mcDNA was obtained with a high purity degree. Comparing this result with the one obtained using the optimized commercial kit (the final nucleic acids mass purified was of 29.73 µg), besides being more than the double, this strategy allowed to obtain purified fractions only composed with sc mcDNA, whereas the commercial kit did no allowed the separation of the mcDNA from the PP. Moreover, comparing the final mcDNA mass recovery using the 60 and 90 cm sepharose column, 36.6 and 94.6, respectively, the mcDNA mass obtained using the bigger column is similar to the one obtained for the mcDNA-primiR-375 (100.84 µg) with the smaller column. So, based on this result it is possible to conclude that adjustments made were efficient to obtain a better mcDNA yield.

In addition, as the use of a 90 cm column allowed to recover more mcDNA in comparison to the 60 cm column, this strategy is suitable to be used for the purification of mcDNA-p53, a vector that when purified with a 60 cm sepharose column presented a low mcDNA yield (Table 7).

4.4 *in vitro* assays

4.4.1 Cellular cytotoxicity evaluation assay

A viability assay was performed in non-cancer cells (human Fibroblasts (hFib)) and cervical cancer cells (CaSki), using the resazurin method in order to determine whether the vectors in the study had any toxic effect towards the cells once transfected.

Resazurin is a blue dye, itself with low fluorescence until being irreversibly reduced to resofurin, which has a pink color and is highly fluorescent, used as an indicator in cell viability assays once the reduction of the resazurin only happens when the cells are viable, after a period of incubation of 4 hours[79].

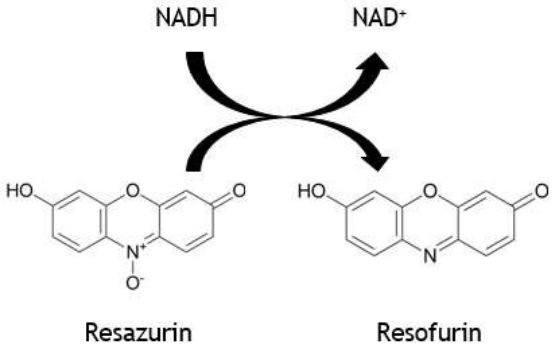

Figure 18: Structure of resazurin substrate and the pink fluorescent resofurin product resulting from the reduction in viable cells (adapted from Riss and co-workers)[79].

The assay performed with hFib is particularly important once it will indicate if the mcDNA vectors are toxic or not to the cells, since it is not expected a therapeutic effect from the mcDNA encoded genes in these non-cancer cells. On the other hand, the CaSki cells were used to try to understand if a decrease in cellular viability would be observed, indicating a possible therapeutic effected induced by the mcDNA vectors in study. The cytotoxicity results in Figure 19 were calculated in relation to the negative control (non-transfected cells), established to 100 %.

The results obtained for the hFib, Figure 19 (A), cellular viability slightly decreases in the first 36 hours. However, from 36 hours until the end of the assay, hFib seem to be capable of recover from the viability diminish, being cellular viability almost fully restored at 72 hours. Thereby, these results suggest that the vectors do not cause a cytotoxic effect, once cellular viability presented always values superior to 80 % in spite of the transfection period

(evaluated from 24 to 72h), not existing significant differences in comparison to the respective negative control.

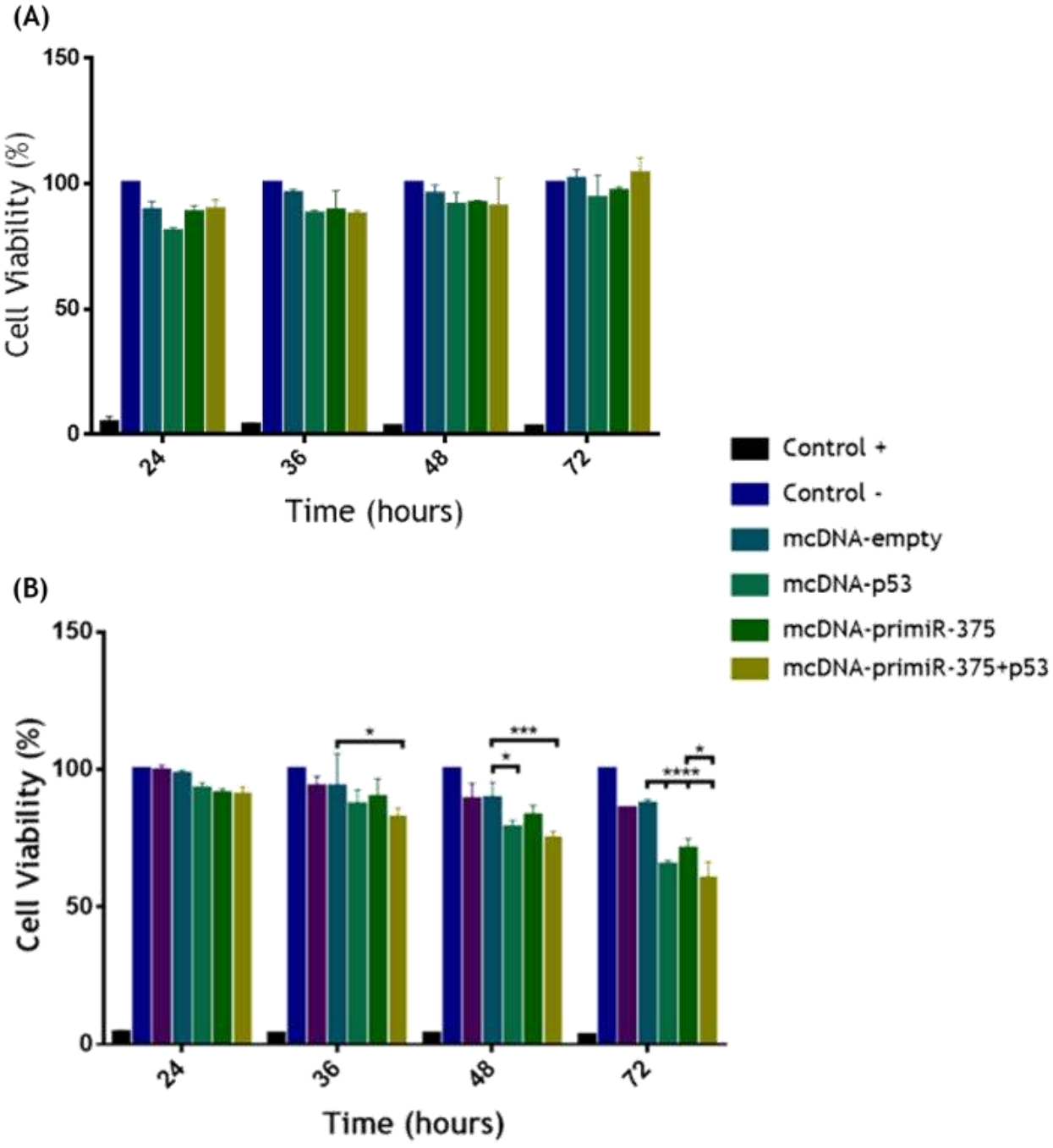

Figure 19: Cell viability percentage in comparison to the negative control (non-transfected cells) in human fibroblasts (A) and CasKi (B) cells, after transfection with mcDNA-p53, primiR-375 and primiR-375+p53 vectors during 24, 36, 48 and 72 hours. The assays were performed for a n = 3 and the statistical analysis was performed using one-way ANOVA with GraphPad software (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

It is also important to highlight that the cellular viability for the cells transfected with mcDNA-empty vector decreases in comparison to the non-transfected cells. This loss of

cellular viability is similarly to the on observed in cells transfected only with the transfection reagent, suggesting that the observed cytotoxicity may be related with the transfection reagent and not with the mcDNA vector transfection process.

On the other hand, through the analysis of Figure 19 (B), it is possible to verify that the cellular viability in the CasKi cells diminishes after the transfection with the studied vectors through the time, beginning to be especially evident after 36 hours. At this time, the cells transfected with mcDNA-primiR-375+p53 present the lower cellular viability in comparison to the non-transfected ones. After 48 hours of transfection, the mcDNA-p53, mcDNA-primiR-375 and mcDNA-primiR-375+p53 diminish to 78%, 83% and 75%, respectively, and after 72 hours, they decrease to 65%, 71% and 60%. These results indicate that throughout the transfection period, the cells transfected with mcDNA-primiR-375+p53 show the lower cellular viability. Moreover, at 72h, mcDNA-primiR-375 and primiR-375+p53 vectors presented a significant difference ($P < 0.05$). These results suggest that the loss of viability can be due to the silencing of E6 and E7 oncoproteins and the re-establishment of p53 and pRb levels. Once again, a difference in cellular viability is observed between the non-transfected cells and the cells transfected with the mcDNA-empty. A cytotoxicity assay using only the commercial transfection reagent was performed in order to evaluate if a decrease in cellular viability would be observe. In fact, the cells incubated with the transfection reagent showed a similar behavior like the cells transfected with the mcDNA-empty. Therefore, it is possible to conclude that the decrease observed in cellular viability of the cells transfected with mcDNA-empty is due to the commercial transfection reagent, as well as, the effect observed with other mcDNA vectors is due to the encoded gene and not with the mcDNA transfection procedure.

Overall, these results indicate that all the mcDNA vectors encoding a therapeutic gene present an influence in the cell viability of cancer cells. p53 is a responsible for regulate the expression of genes encoding regulators of the cell cycle, DNA repair machinery and apoptosis[12]. On its turn, the expression of miRNA-375 decreases during HPV-mediated cervical transformation[52] and interestingly, was shown to suppress the expression of multiple host cellular and viral oncogenic factors including the E6 associated protein (E6AP) and the E6 and E7 oncogenes of HPV 16 and 18[27]. Thus, miRNA-375 may contribute to the intracellular surveillance to prevent the oncogenic activity and is therefore suggested to play a tumour suppressive role, especially in HPV-associated cancers[48]. The coordinated action of these two genes may explain why the cells transfected with mcDNA-primiR-375+p53 presented the lower cellular viability, independently of the transfection period, suggesting that a therapeutic effected is being obtained with the use of the mcDNA vectors.

4.4.2 Proliferation assay

The cellular proliferation assay was performed in order to evaluate if cellular viability would decrease in comparison to the non-transfected cells (Control -), once the desired therapeutic effect should depend on the block of cellular growth and, as a consequence, its death. CaSki cells were transfected with mcDNA vectors in study and then, they were recovered using trypsin and counted using the trypan blue exclusion technique (viable cells would remain white and the ones that were dead would become blue) after 0, 24, 36, 48 and 72 hours of transfection. The graphical representation of the viable cells throughout proliferation assays is presented in Figure 20.

After 24 hours of transfection, it is possible to observe a reduction in the number of viable cells transfected with the mcDNA vectors in study. At 36, 48 and 72 hours, the number of viable cells transfected with the mcDNA vectors remain lower in comparison to the non-transfected cells. In the experiments with mcDNA-primiR-375, the cellular viability decrease might be associated with the silencing of E6 and E7 oncoproteins, and has a consequence, the re-establishment of tumour suppressor p53 and pRb proteins levels, which can trigger cell cycle arrest[6]. Jung and co-workers[27] validated that an increment of miRNA-375 levels led to the silencing of E6 and E7 oncoproteins and, as a consequence, to the re-establishment of p53 and pRb levels in HPV 16 and 18 cell lines. In the experiments with mcDNA-p53, the cellular viability decreases, that could be associated with the cell supplementation of tumour suppressor protein p53 levels induced by the mcDNA vector, leading once again to cell cycle arrest[12]. Costa and co-workers[80] research show an inhibiting and decrease of cellular proliferation in HPV cell lines transfected with DNA vectors throughout time after transfection.

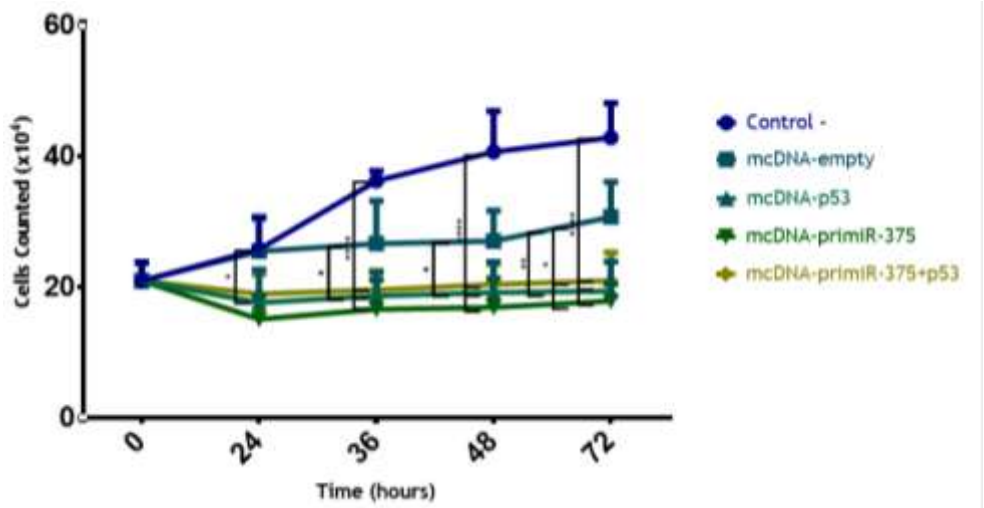

Figure 20: Cell growth assay in CaSki cells after transfection with mcDNA-p53, primiR-375 and primiR-375+p53 vectors during 0, 24, 36, 48 and 72 hours. The assay was performed for a n = 3 and the statistical analysis was performed using one-way ANOVA with GraphPad software (*P < 0.05; **P < 0.,01; ****P < 0.0001).

Similarly, to the results obtained for the cytotoxicity assay in CaSki cells, Figure 19(B), the results for the cellular proliferation showed a decrease of cellular viability when used the mcDNA-p53, mcDNA-primiR-375 and mcDNA-primiR-375+p53. Once again, the cells transfected with mcDNA-empty vectors showed a decrease on their viability, that as discussed previously in the cytotoxicity assay performed on CaSki cells, can be due to some toxicity induced by the use of a commercial transfection reagent.

4.5 Western-Blot

The purpose of using the mcDNA vectors encoding for primiR-375 and p53 is to re-establish the levels of p53 and pRb tumour suppressor proteins as well as inhibit the expression of the E6 and E7 oncoproteins. To determine if these vectors were able to restore and inhibit the target proteins, a Western-Blot assay was performed to firstly evaluate the influence of mcDNA vectors on the p53 levels. So, non-transfected and transfected cells after 48 hours of transfection were submitted to protein extraction and quantification for posterior Western-Blot analysis (Figure 21).

Through Figure 21 analysis, and as expected the non-transfected CaSki cells do not show the presence of the p53 tumour suppressor protein, once this cell line has more HPV copies and therefore present a more malignant phenotype. This absence of p53 was also noticed by Ding and co-workers[81].

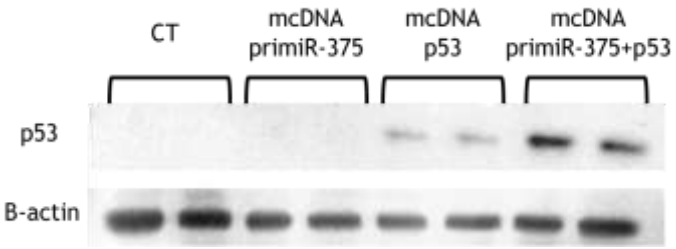

Figure 21: Western-Blot for the detection of tumour suppressor p53 (53 kDa) protein and respective normalization with housekeeping protein ß-actin (42 kDa) after 48h of transfection in CaSki cells. CT – non-transfected cells.

The lanes corresponding to the cells transfected with mcDNA-primiR-375 seem to have an extremely faint band, hence being necessary to perform a longer transfection period so that the effect of the p53 levels recovery due to the blockage of E6 oncoprotein function (inducing the p53 degradation). A 72 hours period of transfection may be ideal to evaluate this effect.

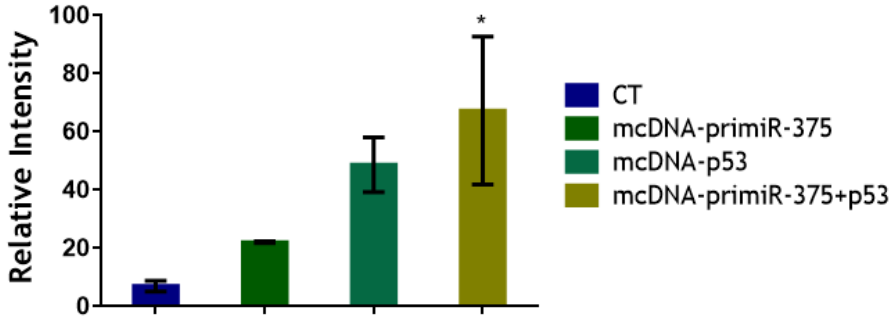

Figure 22: Graphical representation of the normalization of p53 protein expression in relation to ß-actin (p53/ß-actin) relative intensity after 48 hours of transfection. The assay was performed for a n = 2 and the statistical analysis was performed using one-way ANOVA with GraphPad software (*P < 0.05).

Analyzing the Figure 21, it is possible to observe that there is no expression of p53 in the non-transfected cells and those transfected with mcDNA-primiR-375 and an increment of the levels of this protein in the cells transfected with mcDNA-p53 and mcDNA-primiR-375+p53. The increment of p53 expression level is more evident in the cells transfected with the mcDNA encoding both for the miR-375 and p53 (Figure 22). This result is in agreement with the literature and the mechanism of action of the oncoprotein E6 in cervical cancer cells, that is responsible for targeting tumour suppressor p53 for degradation[26].

Comparing the supplementation of p53 levels in cells transfected with the mcDNA-primiR-375 and the mcDNA-p53 and mcDNA-primiR-375+p53, it is notorious that cells transfected with mcDNA vectors encoding for the tumour suppressor p53 present a faster increment of this therapeutic protein levels. However, it is also important to highlight that some degradation may occur for the cells transfected with mcDNA-p53 due to the presence of HPV oncoprotein E6. This may explain the difference observed in the level of p53 in cells transfected with mcDNA-p53 after 48 hours of transfection in comparison to those transfected with mcDNA-primiR-375+p53 that simultaneously seems to inhibit the oncoprotein and increases the tumour suppressor levels (Figure 21).

Due to the fact that the cells transfected with mcDNA-primiR-375+p53 presented a higher expression level of the tumour suppressor protein p53, another Western-Blot comparing only non-transfected and transfected cells with mcDNA-prmiR-375+p53 was performed, Figure 23, and at this time, also the pRb was evaluated. Once again, the non-transfected cells did not show the presence of tumour suppressor p53 proteins contrarily to the CaSki cells transfected with mcDNA-primiR-375+p53. Both for the non-transfected and the transfected cells, it is observed the presence of pRb. Contrarily to p53 that is degraded due to the actions of the oncoprotein E6 and E6AP complex, once the oncoprotein E7 binds to the pRb, it will occur an inhibition of its tumour suppressor role but does not lead to its degradation, the reason why it is possible to observe the presence of this protein in the non-transfected cells.

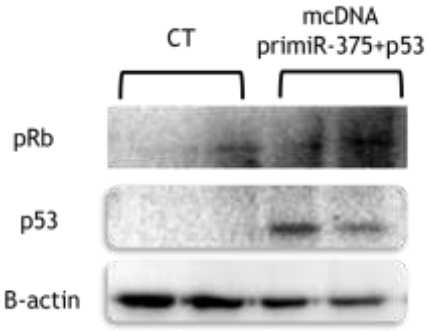

Figure 23: Western-Blot for the detection of tumour suppressor p53 (53 kDa) and pRb (107 kDa) proteins and respective normalization with housekeeping protein β-actin (42 kDa) after 48h of transfection in CaSki cells. CT – non-transfected cells.

After confirming the expression of pRb and p53 proteins, the normalization of the results was performed with a housekeeping protein, the β-actin. Once this protein is involved in cellular maintenance functions, it is present in all the human cells, at relatively constant levels.

Figure 24 represents the identification of E6 oncoprotein in non-transfected CaSki cells (control) using 30 and 50 µg of protein. This is just a preliminary result, once some optimizations are currently being made, in order to identify the oncoproteins. Several concentrations of the protein extract obtained after the CaSki cells lysis were prepared to find out the most suitable for the detection of the desired protein. In the future, it will be necessary to compare if the expression levels of E6 oncoproteins decrease in the cells transfected with the mcDNA vectors.

Through Figure 24 analysis, both total protein concentrations evaluated (30 and 50 µg) and the followed procedure seem to be suitable for the identification of the E6 oncoprotein. As such, for further Western-Blot analysis, a concentration of 30 µg of total protein will be chosen, once it is the same used to identify the presence of tumour suppressor proteins p53 and pRb.

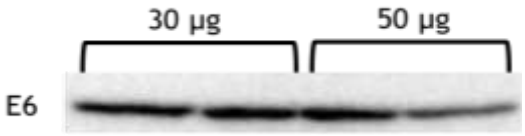

Figure 24: Western-Blot for the detection of E6 oncoprotein after 48h of non-transfected CaSki cells, using 30 and 50 µg of protein.

Chapter V – Conclusions and future perspectives

The high prevalence of HPV infections and the growing number of cervical cancer cases leads to the need of finding efficient treatments for this disease. The DNA based therapies have been considered as an innovative strategy, appearing the mcDNA vector as a promising alternative to the conventional and well-studied plasmid DNA.

So, this work aimed to produce and purify different mcDNA vector encoding for primiR-375, p53 and both genes in order to restore tumour suppressor protein levels and silence HPV oncoproteins. To achieve the proposed goals, it was necessary to first produce and purify the biological active mcDNA conformation, the supercoilied (sc) isoform. After exploring two different chromatographic techniques for the purification of mcDNA, the results obtained with anion exchange chromatography demonstrated that in spite of the final sample obtained without RNA, this sample presented genomic DNA and parental plasmid, therefore not being the most suitable technique for the mcDNA purification, due to its low mcDNA proportion. On the other hand, the results obtained using size exclusion chromatography allowed an efficient separation of the desired mcDNA from the genomic DNA, parental DNA plasmid and RNA. As such the purity degree of the mcDNA vectors purified using size exclusion chromatography may be evaluated in order to understand if this strategy allows the purification of mcDNA that fits the regulatory agencies criteria.

Through the cellular proliferation assay it was possible to verify that the mcDNA-p53, mcDNA-primiR-375 and mcDNA-primiR-375+p53 inhibit the cellular proliferation. The same trend was observed in the cellular cytotoxicity results performed in CaSki cells. Furthermore, through the analysis of the cytotoxicity results, it is possible to observe that the mcDNA-primiR-375+p53 is responsible for the highest cellular viability decrease. The results from the cytotoxicity assay in human Fibroblasts showed that the mcDNA vectors in study do not cause significant cytotoxic effects, as cellular viability is always higher than 80%, despite the period of transfection. Finally, the increment of p53 protein levels was evaluated in cells transfected with the mcDNA vectors by Western-Blot, showing that mcDNA-primiR-375+p53 vectors is responsible for the higher expression level of tumour suppressor protein p53. Considering the results obtained in the different *in vitro* assays, it is possible to conclude that the mcDNA enconding both for p53 and primiR-375 demonstrated to have the highest cellular viability decrease and the highest tumour suppressor p53 protein expression level increment. These

results indicate that the coordinated action of both genes allowed to improve the therapeutic effect faster than the one obtained using the vectors separately.

In the future, taking into consideration the therapeutic potential of the mcDNA-primiR-375+p53, the oncoproteins levels will need to be evaluated as well as proved the presence of the tumour suppressor proteins and oncoproteins through other proteomic techniques to confirm the results observed in western bolt analysis. It will be also interesting evaluate if the mcDNA-primiR-375, mcDNA-p53 and mcDNA-primiR-375+p53 vectors will induce the intrinsic apoptosis pathway though the evaluation of Bax protein levels in cells transfected with the mcDNA vectors, via western blot or other complementary proteomics techniques.

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